IDENTIFYING OF MORE POTENT AND EFFICACIOUS ANALOGS OF THE NOVEL HOST-DERIVED IMMUNOSTIMULANT EP67

By

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أُهديها بِكلِ إجلالٍ واشتياق إلى أُمي و أُبي، وبِكل مودةٍ وحُب إلى زوجتي وبناتي، وبِكل احترامٍ وتقدير إلى إِختي وأَخواني، وبِكل اعتزاز وفخر إلى موطِني بلادِ الحرمين

This dissertation is dedicated with all love and gratitude to my family

'I will love you forever'

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We dedicate this work to the memory of Dr. Sam Sanderson, the inventor of EP54 and EP67. His larger-than-life personality, tenacious approach to research, and unwavering friendship will be greatly missed.

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IDENTIFICATION OF MORE POTENT AND EFFICACIOUS ANALOGS OF THE NOVEL HOST-DERIVED IMMUNOSTIMULANT EP67

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EP67 is a decapeptide agonist of C5a Receptor 1 (C5aR1/CD88) based on the C-terminus of human C5a that selectively activates mononuclear phagocytes over neutrophils to stimulate protective innate and adaptive immune responses while potentially minimizing neutrophil-mediated toxicity. Pro^{7} and *N*-methyl-Leu^{8} (nme-Leu^{8}) induce structural changes within EP67 that increase potency and selective activation vs. neutrophils. *Cis*/*trans* isomerization at Pro^{7}, however, likely limits the activity of EP67 and the low coupling efficiency between Pro^{7} and nme-Leu^{8} increases scale-up costs for clinical use. Thus, the goals of this project were to (i.) develop a clinically relevant, high-throughput assay for screening immunostimulant activity in primary human mononuclear phagocytes (monocytes, unpolarized (M0)-monocyte-derived macrophages, monocyte-derived dendritic cells) and neutrophils and (ii.) determine whether replacing Pro^{7} with cyclohexylalanine and/or nme-Leu^{8} with leucine adversely affects EP67 potency and efficacy in human mononuclear phagocytes and selective activation vs. human neutrophils. We found that, depending on the mononuclear phagocyte and cytokine, EP67 analogs (i.) had similar or lower EC_{50} and similar, increased, or decreased E_{MAX} for IL-6 and TNF-α secretion from mononuclear phagocytes 24 h after treatment and (ii.) EP67 analogs does not affect selective activation of

mononuclear phagocytes vs. neutrophils. Thus, replacing Pro^{7} and/or nme-Leu^{8} with amino acids that induce similar structural changes affects the activity of EP67 depending on the mononuclear phagocyte and cytokine but is an amenable approach for future analogs.

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List of Abbreviations

APCs: Antigen presenting cells

BCG: Bacillus Calmette and Guerin

CCM: Complete culture medium

CDI: Complement-derived immunostimulants

CLRs: C-type lectin-like receptors

CpG Motifs: Cytosine-phosphate-guanosine motifs

CR3: type 3 Complement receptor

DCs: Dendritic cells

DD: Death domain

HIV-1: Acquired immunodeficiency virus

HPV: Human papillomavirus

Hsps: Heat shock proteins

IFN-γ: Interferon- γ

IgG: Immunoglobulin G

IL: Interleukin

IRAK: Interleukin-1 receptor-associated kinase

LacCer: Lactosylceramide receptor

LAK: Lymphokine-activated killer cells

LPS: Lipopolysaccharides

MAP kinases: Mitogen activated protein kinases

MHC: Major histocompatibility complex

MPL: monophosphoryl lipid A

MyD88: Myeloid differentiation 88

NFκB: Nuclear transcription factor kappa B

NK cells: Natural killer cells

NOD: Nucleotide oligomerization domain like receptors

ODN: Oligodeoxynucleotides

PAMPs: Pathogen-associated molecular patterns

pDC: plasmacytoid Dendritic Cell

PRRs: Pattern recognition receptors

RIG-1: Retinoic acid-inducible gene-1

Th1: T helper cells 1

Th2: T helper cells 2

TIR domain: Toll-interleukin receptor domain

TLR: Toll like receptor

TNFα: Tumor Necrosis Factor α

TRAF6: TNF receptor associated factor 6

CHAPTER 1

GENERAL INTRODUCTON

1 GENERAL INTRODUCTION

1.1 Chance and necessity: The first attempt of immunostimulants

The concept of immunostimulants was initiated early last century when Dr. William Coley, a renowned surgeon at New York Memorial hospital, utilized killed streptococcus pyogenes and Serratia marcescens (Coley's vaccine) to treat lymphomas, melanomas, and myelomas. His insight was that active defense against microbes reinforced the body's fight against cancer. This observation leads to the origin of the combination of streptococcus pyogenes and Serratia [1]. Initially, Coley and other researchers were using live bacteria; however, the fatal infection caused by these induced bacteria led to the use of inactivated organisms. After the success of using inactivated organisms, several other microbial substances were clinically approved to be used in the treatments of infections and cancers (**Table 1.1**) [2]. However, the use of immunostimulants to enhance the humoral immunity was recognized later, in 1925, when Gaston Ramon observed that giving diphtheria toxoid with the combination of starch, fish oils, and several plants extract can enhance the response of antibodies towards the toxoid in the horses. Following this, after a year, Glenny also observed the same effect with Alum, which was approved later as an adjuvant in a variety of vaccines [3-5].

Immunostimulants are either natural or synthetic substances that are used to induce and stimulate nonspecific activation of the immune system, unless they are associated with antigens (e.g., adjuvants in vaccines). Immunostimulants can amplify different effectors of the immune response, including phagocytosis, complement system, protective secretory antibodies, α- and γ-interferon release, T- and B-lymphocytes, and synthesis of pulmonary surfactant [1,6]. They also support the release of endogenous immune mediators such as cytokines and chemokines that aids in the treatments of several chronic diseases such as infectious diseases, immunodeficiencies, or cancer. Immunostimulants vary based on their origin, mechanism of action, and application (preventive, therapeutic, or adjuvants) [1].

1.2 The impact of immunostimulants

Through the last three decades, there have been intensive researches that are carried out on the development, preparation, experimental, and clinical characteristics of immunostimulants. From the therapeutic point of view, the ability of immunostimulants to stimulate the immune system has a great potential as adjuvant therapy in cancer, chronic infections, immunodeficiency diseases, and vaccines [6,7].

One of the greatest achievements of modern medicine has been the development of antimicrobials for the treatment of infectious diseases [8]. Antibiotic resistance, however, has turned into a significant medical problem. For instance, staphylococcus aureus penicillin resistance has been reported since 1947, four years after the start of large-scale production, and since then, the amount and variety of pathogenic strains resistant to new classes of antibiotics have increased [9,10]. This has made various treatment schemes with antimicrobial drugs less effective. Therefore, the interest in the development of new alternatives for the treatment of bacterial infections has become a global emergency [11]. Combating pathogenic organisms by combined therapies involving appropriate immune response molecules and antimicrobial drugs represents an apparent and successful therapeutic paradigm for the treatment of acute and chronic infectious diseases [12]. Several studies have demonstrated the abilities of different immunostimulants to stimulate host defense mechanisms for the prophylaxis and treatment of diverse viral, bacterial, parasitic, and fungal diseases. The potential of these agents to stimulate the immune response can be used for treatment or as adjuvant therapy of various microbial infections [13].

In the case of vaccination, adjuvants are substances used to increase the cellular/humoral response to a used immunogen to prevent infectious diseases. In

general, among the mechanisms that adjuvants use to boost the immune response are the "deposit" effect, antigen presentation, antigen distribution, immune modulation/activation, and induction of cytotoxic lymphocytes [14,15]. Adjuvants can significantly improve the protective immune response, and today their in-depth knowledge is the key to the development of better vaccines. The classic adjuvant at present is the aluminum salts (aluminum phosphate and aluminum hydroxide), which discovered in the first half of the twentieth century [16]. These adjuvants, in addition to being deposited in a particulate manner in the tissues and inducing phagocytosis of the antigen, activate macrophages, stimulate the T helper 2 response (Th2) and are frequently used when the immunological effector sought is antibodies, since they stimulate the production of IgG 1 [16,17]. They do not significantly stimulate cellular immunity, since the adjuvant adsorbs to proteins, and the physicochemical conditions of antigen and adjuvant must be considered in this process. Recently, new aspects of the mechanism of action of aluminum hydroxide have been described, including the activation of the innate immune system and the production of IL-1, through the activation of the NPLR3 protein complex in monocytes and induction of immunogenic dendritic cells [18]. AS04 is another approved adjuvant that is made by a combination of aluminum salt and a Toll-like receptor 4 agonist (TLR4), monophosphoryl lipid A (MPL). MPL is

produced by detoxification and purification of lipopolysaccharides (LPS), originating a non-toxic molecule with immunostimulatory properties of TLR. The AS04 system causes a local transient response of cytokines that increases the activation of monocytes, dendritic cells, and antigen-specific T cells. This adjuvant system has been incorporated into some vaccines, such as Cervarix, approved for use against human papillomavirus and in the hepatitis B surface antigen vaccine (Fendrix), developed for patients with kidney disease and high risk of hepatitis B [19,20].

Also, natural, synthetic, or modified adjuvants are evaluated, which can be used to stimulate the immune response against specific pathogens. Such is the case of adjuvants MF59, an emulsion of squalene-tween 80 and sorbitan trioleate, which is a natural organic compound originally extracted from the shark's liver. MF59 is approved in Europe and is present in various vaccines, such as influenza. Another example is AS03, oil-water emulsion, and vitamin E and AS02 containing MPL and QS21 oil-water emulsion [21,22].

1.3 A turning point: Better understanding of immunology and its impact on the development of immunostimulants

For decades, the discovery and development of novel immunostimulants have been based on observations and experiments, without explicit immunological knowledge of the mechanism that causes the immunostimulatory effect. However, the discovery of the innate immune system's ability to recognize molecular pathogen-associated molecular patterns (PAMPs), via specialized receptors, pattern recognition receptors (PRRs), has had significant consequences for the development of new immunostimulants. PRRs are innate immunity receptors being involved in many immunological and immunopathological processes. Recently, several new families of PRRs have been identified including Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), nucleotide oligomerization domain (NOD) like receptors (NLRs), and Retinoic acid-inducible gene-1 (RIG-1) like receptors (RLRs) [23-25].

The innate immune system acts as a sentinel: the cells that are activated on contact with the PAMPs (especially the dendritic cells), which triggers their maturation, which enables them to efficiently present the antigen to the T lymphocytes, in the context of major histocompatibility complex (MHC system) [26]. The recognition of PAMPs by PRRs (especially TLRs) generates a cascade

of activation within the immune cells. This cascade results in the activation of a nuclear transcription factor (NFκB), which allows the transcription of inflammatory mediator genes such as proinflammatory cytokines (IL-1,IL-6, and TNFα), chemokines, adhesion molecules and cyclooxygenases-2; leading to attract more T cells to the point of inflammation and cause them to differentiate into polarized effectors. This discovery naturally led to the use of PAMPs as immunostimulants [27].

1.4 Classification of immunostimulants

Most immunostimulants reviews during recent decades followed different criteria to classify the immunostimulants. In this section, immunostimulants are classified based on their origin. Also, the mechanism of actions of these substances will be highlighted according to the cell induction capacity and the type of response they initiate.

1.4.1 Microbially-derived immunostimulants

1.4.1.1 Bacterial lipopolysaccharides endotoxins

The lipopolysaccharides of Gram-negative bacteria are prototype of PAMPs. LPS contains a fraction called lipid A, composed of fatty acids linked by steric bonding to N-acetylglucosamine molecules. Another fraction is the R region,

which is composed of hexoses, and the third fraction is the so-called O antigen, which is specific for each bacterial species [28]. These molecules are essential to the bacterial wall structure and are well-known activators of the inflammatory response. The PRRs involved in the recognition of LPS has long remained unknown, however, Poltorak and colleagues have identified the role of TLR4 as LPS-sensing receptor. The recognition of LPS via TLR4 induce a signaling pathway involving the myeloid differentiation 88 (MyD88) cytoplasmic molecule that contains a Toll-Interleukin receptor domain (TIR domain) and a death domain (DD). After recruitment, the MyD88 molecule induces the recruitment and the activation of the Interleukin-1 receptor-associated kinase 1 and 4 (IRAK kinases 1 and 4), responsible for the activation of the molecule TNF Receptor Associated Factor 6 (TRAF6). This then activates the NFκB, which is involved in the induction of many genes, mainly pro-inflammatory cytokines. Moreover, activation of TLR4 induce the activation of the TRIF molecule, a step required to produce type I interferon: TRIF activates the IRF3 transcription factor, and it is binding on the promoter of the IFNβ gene [29-31]. Several synthetic analogs of the constituents of biologically active bacterial cell walls, such as lipid A, part of the LPS of gram-negative bacteria, and synthetic muramyl dipeptide derived from bacterial peptidoglycan have prepared [32,33].

1.4.1.2 Unmethylated Cytosine-Phosphate-Guanosine Motifs (CpG Motifs)

The synthetic oligonucleotides (ODN) of the CpG are single-stranded DNA sequences containing CG or CpG motifs. There are several classes of CpG-like ODNs (A, B, C, and P) that differ in their structure and immunostimulatory effect [34]. The CpG type ODNs are potent immunostimulants capable of activating most innate immunity cells such as natural killer cells (NK cells), T lymphocytes, B cells, monocytes, macrophages and DCs by detecting the intracellular presence of the cell DNA of phagocytotic pathogens [34,35]. The CpG type ODNs are predominantly recognized via the TLR9 receptor, which is located in endosomes and is primarily expressed by plasmacytoid Dendritic Cell (pDC) and B cell lymphocytes in humans, and in addition by the myeloid lineage (macrophages, monocytes, myeloid dendritic cells) in mice [36]. Human TLR9 would also be expressed in monocytes and macrophages but at a lower level [37].

Intracellular activation after TLR9 binding leads to the activation of mitogen activated protein kinases (MAP kinases) and NFκB pathways [36]. The attachment of CpG-like ODD to TLR9 in B cells triggers the secretion of cytokines such as IL-6 and IL-10, cell proliferation, and expression of MHC class II molecules [38]. Type A ODDs strongly activate NK cells and pDCs and cause strong interferon- γ (IFN-γ) secretion by pDCs while type B ODNs strongly activate B-cells and pDCs. The

C-type ODNs combine the properties of the two previous types. P-type ODNs stimulate B-cells, activate pDCs and induce strong IFN-γ secretion by pDCs such as type-A ODNs [36].

CpG-type ODNs are used either alone to promote an immune response, or in combination with other treatments such as monoclonal antibodies, chemotherapy, radiotherapy to increase its effectiveness, or as adjuvants in a vaccine. Several studies on solid tumor models have shown the effect on tumor development of the CpG-type ODN used alone [39-41]. For instance, a murine model of neuroblastoma has shown that a daily injected of CpG-type ODN near the tumor led to tumor regression in half of the animals, and the appearance of a prolonged protective immune response. Moreover, the combination of therapeutic monoclonal antibodies with CpG-type ODN increases the cytotoxicity relayed by antibodies by stimulating macrophages and NK cells. The combination of Trastuzumab (anti-Her2neu antibody) with CpG-type ODN, for instance, has shown to inhibit murine tumor growth in 96% of the human cell lines (MCF-7 and BT474 xenografts) [42]. In an orthotopic model of a pancreatic tumor, the combination of Gemcitabine and ODN CpG type allows to delay tumor development and increase the survival rate and reduces the spread of the tumor in the peritoneal cavity than the chemotherapeutic treatment alone [43]. Many

other mouse model studies have also shown improved efficacy of therapeutic treatments when combined with CpG-like ODNs. As a result, several clinical trials using CpG-type ODDs alone or in combination are underway [44].

1.4.1.3 Bacillus Calmette and Guerin (BCG) and active components of the mycobacterial cell wall

BCG is a highly complex immunogen that induces a cellular-type immune response [1,45]. In 1921 Calmette and Guerin, after 13 years and 230 cultivation passes of a strain of Mycobacterium bovis, obtained a non-pathogenic but immunogenic bacillus, whose use managed, with varying efficacy, to reduce the incidence of tuberculosis and tuberculous meningitis for its systemic protective immunostimulatory effect [1].

Although BCG has been used successfully for many years, details of its antineoplastic mechanism of action remain unknown. It is thought that a wide variety of immunocompetent cells, as well as uroepithelial cells and even the same bladder neoplastic cells, participate in the immune mechanism that occurs during intravesical therapy with BCG [45]. The anti-tumor effects of BCG appear to be related to immunological mechanisms, which are reflected by a transient increase in several cytokines and the presence of immunocompetent leukocytes activated

in the urine within 24 hours after instillation [46]. Some studies have revealed that CD4 + T cells and monocytes act as accessory cells in cell-mediated cytotoxicity induced by BCG [47]. Certainly, another large number of molecules and cells must participate in BCG-induced immune stimulation, such as the family of adhesion molecules, heat shock proteins, toll-like receptors, nitric oxide, dendritic cells and their subpopulations, the regulatory T cells and the recently described Th17 cells [48,49].

1.4.1.4 β-glucans

β-glucans are polysaccharides formed from units of glucose linked together in β-1,4 and β-1,6. They differ in their structures (length and ramifications). These are compounds of the walls of fungi, yeasts, bacteria, algae, and plants, known in traditional medicine as immunostimulants [50,51]. Indeed, fungi and algae have branches rather than 1 to 6, while bacteria and plants have more side chains in 1 to 4. The existing multiple β-glucans exhibit differences in immunostimulatory properties, and their polydispersity explains the complexity of purifying and studying them [52].

Macrophages can internalize β-glucans, fragment them in endosomes, and the released fragments of soluble β-glucans are then recognized by different

immune cells with specific receptors [53]. The primary receptors of known β-glucans are lectins such as dectin-1 expressed on the surface of macrophages, neutrophils, and dendritic cells and integrins such as the type 3 complement receptor (CR3) expressed on the surface of NK cells and neutrophils [54,55]. They can also be recognized by the scavenger receptor and by the LactosylCeramide receptor (LacCer) expressed on the surface of neutrophils. CR3 recognizes opsonized or non-complemented β-glucans and directly induces phagocytosis. Thus, by binding to this receptor, β-glucans would cause a CR3 conformational change allowing better exposure of the iC3b binding domain, thus facilitating the interaction between integrin and its ligands and thus the activation of NK cells and neutrophils involved in the elimination of cancer cells [56].

Several clinical trials have demonstrated that the combination of chemotherapy with β-glucans promotes the activation of CR3 on NK cells and increases the effectiveness of treatment [57]. A study has shown that a group of mice inoculated with tumor cells had a significant slowing down of the increase in tumor size with a treatment combining the antitumor monoclonal antibody and the β-glucans compared to treatment with the antibody alone or β-glucans alone. Also, 100 days after tumor implantation, a significant percentage of survival of mice

treated with monoclonal antibodies and β-glucans was observed, compared to other types of treatments [58].

1.4.2 Synthetic-derived immunostimulants

1.4.2.1 Levamisole (Ergamisol):

Levamisole was initially developed as an anthelmintic and widely used for the treatment of gastrointestinal and tissue helminths [59]. Various studies have been carried out to demonstrate the immunostimulatory activity of levamisole, some of them with conflicting results. This drug, when administered in combination with the canine parvovirus vaccine, boosted antibody production, increased phagocytic activity and stimulated lymphocyte proliferation activity and similarly potentiated the protective antibody response to hepatitis B vaccination in patients with hemodialysis [60]. The effect of levamisole on cellular immunity is mainly on anaerobic T lymphocytes, macrophages and polymorphonuclear leukocytes [61]. In 2000, the US FDA was withdrawn Levamisole from the market due to its ability to cause serious adverse effects, including agranulocytosis [62]. Interestingly, levamisole has been found as an adulterant in cocaine and can lead to a variety of adverse effects in individuals using this drug [63].

1.4.2.2 Isoprinosine

Isoprinosine is an antiviral, antitumor, and immunostimulatory agent with superior properties to levamisole. Isoprinosine works as a thymomimetic hormone, promoting the production of a factor that activates the functions of the various cytokines and increases the production of IL-1, IL-2, and interferon. It facilitates the differentiation of T lymphocytes and the increase of its receptors [64]. It is also able to increase the phagocytosis function of macrophages and cellular and humoral responses [65]. It is used in the treatment of viral infections, such as herpes simplex and encephalitis of viral origin [66].

1.4.3 Thymus-derived immunostimulants

Thymic hormones are soluble substances obtained from the thymus (Thymopoietin, Thymostimulin, Thymosin, Thymulin), which were attributed with a favorable effect of maturation of T lymphocytes in this organ. This hormone is considered to induce maturation of pre-T cells; therefore, the purified hormone has therapeutic application potentials in various states of T-cell deficiency [67]. A recombinant peptide derived from this hormone; thymosin alpha-1 induces increases in the production of IL-2 and the expression of IL-2 receptors in T lymphocytes. This peptide is being studied in clinical experiments for the treatment of cancer and chronic active hepatitis [68]. In general, thymic hormones may have

utility in primary and secondary immunodeficiencies causing differentiation of non-functional precursors in immunocompetent T cells. Among the side effects observed with these factors are allergic reactions, especially with poorly purified preparations [69].

1.4.4 Host-derived immunostimulants

1.4.4.1 Exogenous Cytokines

Cytokines are specific non-antigen soluble proteins, mostly produced by leukocytes that regulate immune system cells and inflammatory processes [2]. These regulatory molecules are involved in the protective response against neoplasms and infections [2]. Although some cytokines are produced by mononuclear cells, either constitutively or in response to activation signals, their induction has been shown to be diminished in patients with chronic infectious diseases so they could benefit from the exogenous administration of these molecules [70]. When cytokines are used to induce immune response, the local advantages are 1) generate high concentrations of cytokines locally, similar to the body's response against foreign antigens; 2) take advantage of the paracrine effects of cytokines in a sustained way that activates the immune system [70,71]. Some cytokines stimulate the production of others, therefore that they interact,

either synergistically or antagonistically and thus their local and systemic effects are intimately involved in the control of host infections [70].

Advances in the understanding of the role of cytokines in inflammatory and immune disorders have led to the development of cytokine-based therapies. These molecules, their receptors and their signaling pathways, are promising candidates for therapeutic participation [71]. The precise understanding of the critical balance between pro-inflammatory and regulatory cytokines in microbial infections is essential to be able to use and regulate with certainty and safety the beneficial effects that are sought through cytokine therapy, thus exploiting its great potential to increase resistance against various pathogens [72]. T helper cells 1 (Th1) are crucial for orchestrating cytokine responses and of utmost importance for the elimination of infectious diseases. These cells produce IFN-γ, TNF-β, and IL-2 required for the effective development of the cell-mediated immune response against intracellular microorganisms [73]. On the other hand, Th2 produce IL-4 and IL-5 that enhance humoral immunity to T cell-dependent antigens and immunity against helminth infections [73]. Therefore, patients in whom cytokine production is altered or in those who carry out chemotherapy treatments, which causes an increased susceptibility to infections, may benefit from exogenous cytokine therapy [74].

Interferons are cytokines initially described in 1957 with an action that interfered with viral replication. They were initially classified according to the cell that secreted them into IFN-α or leukocyte (produced by mononuclear phagocytes), IFN-β, or fibroblastic (produced by fibroblast) and IFN-γ (produced by lymphocytes and NK cells). They are currently classified according to their receptors and the homology of their sequences. Interferons such as IFN-α and INF-β are classified as type I, and IFN-γ is the only one classified as type II [75].

The mechanism of action of IFN-α and INF-β is related to inhibition of replication and viral spread. It also activates and regulates the expression of the MHC I. IFN-γ stimulates and activates macrophages, neutrophils and NK cells in their phagocytic and cytotoxic functions, in addition to inciting the expression of molecules of the MHC I [75]. IFN-α (INTRON A) is indicated in the treatment of hepatitis B and C and as an adjuvant in myeloma [76]. On the other hand, IFN-γ (ACTIMMUNE) is recommended as adjuvant therapy in chronic granulomatous disease, a primary immunodeficiency defect in phagocytic function in neutrophils and malignant osteopetrosis [77].

Interleukin 2 is a potent activator of T lymphocytes, activates NK cells, macrophages, B lymphocytes and stimulates the release of several cytokines. Its

antitumor effect is mediated by promoting the activation of NK cells, lymphokine-activated killer cells (LAK) and other cytotoxic cells, as well as the induction of IFN-γ, TNF-α [78]. The use of IL-2 in therapy cancer gene has been widely used in various preclinical tumor models, including hepatocellular carcinoma, head, neck carcinoma, sarcomas, plasmacytoma, breast carcinoma, lymphomas, and in cervical cancer [79]. Particularly, the effect IL-2 adjuvant in cervical cancer has been tested in tumor models associated with human papillomavirus (HPV). It has been shown that treatment with the recombinant protein of IL-2 gene, administered intratumorally, significantly reduces the progression of HPV-associated tumors and inhibits the formation of recurrent tumors after being removed by surgery [80].

Cytokines have also been described as a double-edged sword, as they have been attributed to both beneficial and harmful effects. Capillary filtration syndrome is the main dose-dependent toxicity in cytokine therapy, specifically IL-2. This syndrome is characterized by an increase in vascular permeability accompanied by extravasation of fluids and proteins that produces interstitial edema and organ failure. The pathogenesis of endothelial cell damage is complicated. It may involve the activation or damage of endothelial cells and leukocytes, the release of cytokines and mediators of inflammation, the alteration of cell-cell, matrix-cell adhesion, and function of the cytoskeleton [81,82]. The

efficacy of exogenous cytokines capable of enhancing normal host defense mechanisms may be reduced to immunocompromised patients lacking effector cells or containing disease-related factors that prevent lymphocyte activation.

1.4.4.2 Heat shock proteins

Heat shock proteins (Hsps) are proteins that are synthesized by the body in response to stress (temperature, exposure to heavy metals, infections, etc.). They have been grouped into families according to their molecular mass: Hsps 60, Hsps 70, Hsps 90, Hsps 110. These proteins are part of the family of molecular chaperones, and they associate with peptides or proteins that are not correctly folded. Hsps are used to prevent the accumulation of incorrectly folded proteins. Indeed, heat stress can result in denaturing proteins [83].

The participation of Hsps in infections and immune reactions occurs at many levels. It is therefore not surprising to observe a growing interest in these proteins in the search for new vaccines. For instance, mice vaccinated with a cell line that expresses a mycobacterial Hsps 60 show remarkable protection against lethal doses of Mycobacterium tuberculosis [84]. Moreover, Hsps 70 and Hsps 25 are expressed during the cycle of the acquired immunodeficiency virus (HIV-1) and associate with specific viral proteins. They then serve as a target for NK cells and

the antibody-dependent cytotoxic cellular response; they have therefore been proposed as a "vehicle" (or vector or excipient) for the antigen in the development of HIV-1 vaccines [85]. In the case of autoimmune diseases, it has also been observed that the administration of epitopes of Hsps 60 and Hsps 70 could modulate the autoimmune reaction via immunization involving suppressor T cells or via induction of lymphocyte clone anergy. Self-reactive T involved; this can be used as a preventive as well as a curative treatment [86].

Hsps also play a role in the body's defense against cancer and currently represent a preferred target for the development of cancer therapeutics. Research on the understanding of the immune response to cancer has demonstrated the involvement of some members of the Hsps family in inducing specific protection against cancer. The importance of molecular chaperones in the recognition by the immune system of tumor cells and the subsequent regression of tumors has been demonstrated in animal models [87,88]. Some Hsps (Hsps 90 Grp94 and Hsps 70) have been detected on the surface of tumor cells where they could activate the immune response. These observations first led to the conclusion that they represented antigens recognized by the immune system. It was thus demonstrated that tumor-infiltrating T cells were able to recognize cells expressing Hsps 70, thus suggesting that these specific T cells could support the local antitumor cellular

response [89]. Another group of studies suggests that Hsps 70 and Hsps 90 participate in the antitumor immune response as carriers of tumor antigen peptides and not as antigens themselves [90].

1.4.4.3 Complement-derived immunostimulants (CDI)

The complement system was discovered in the late 1890s. It is described as a serum thermolabile component, which has antimicrobial properties, capable of complementing the action of antibodies. Since then, knowledge has evolved, and it is now established that this system is an integral part of the innate immune system. This system refers to a set of serum proteins that play a crucial role in innate immunity: allowing the host to defend against pathogens, participating in the elimination of their cells under the condition of apoptosis. On the surface of apoptotic cells or the pathogen, the complement system can be cascaded in three ways: the classical pathway, the lectin pathway, or the alternative pathway. These three pathways converge to result in the activation of the complement system, which results in the release of several biologically active peptide fragments C3a C4a, and C5a called anaphylatoxins that have diverse biological functions. These fragments are biologically active cleavage products of the more abundant complement proteins C3, C4 and C5, respectively. Apart from their direct action, each of these pathways can induce inflammatory responses and modulate innate

and adaptive immune responses leading to the elimination of pathogens, immune complexes, and apoptotic cells [91,92].

One of the biological consequences of the activation of the complement system is the release of C3a, C4a, and C5a cationic peptides. Initially, C5 convertase from the classical (C4b2a3b), lectin (C4b2a3b) or alternative (C3bBb3b) pathways cleaves C5 into C5a and C5b. C5a remains in the fluid phase and C5b associates rapidly with C6 and C7 and inserts into the membrane [92]. C5a generated in the lytic pathway has several potent biological activities. It is the most potent anaphylatoxin. In addition to the ability to induce an anaphylactic shock, C5a intervening in the inflammatory response in causing contraction of the muscles, vasodilatation, and the vascularization of vascular permeability. The properties of anaphylatoxin C5a may be partially inhibited or controlled after cleavage of carboxy-terminal arginine by serum carboxypeptidase leading to the generation of C5a-desarg [93].

1.4.4.3.1 The structure and functions of C5a

Anaphylatoxin C5a is a polypeptides molecule that is composed of 74 amino. NMR spectroscopy proved that the molecule is composed of four helices and connected by peptide loops with three disulphide bonds between helix IV and

II, III. There is a short 1.5 turn helix on N terminus, but all agonist activity take place in the C terminus.C5a is rapidly metabolized by a serum enzyme carboxypeptidase B to a 72 amino acid form C5a des-Arg without C terminal arginine [94].

Of the three anaphylatoxins, C5a is more potent and binds to at least two receptors C5aR (CD88) and C5L2. The functional role of C5L2 is enigmatic. C5a is a 74 amino acid peptide with pleiotropic biological functions including smooth muscle contraction, vascular permeability, mast cell degranulation, leukocyte chemotaxis and attraction (**Figure 1.1**) [95]. Early studies involving complement mediated immune regulation showed that C5a potentiates antibody and antigen-induced T cell proliferative responses in vitro possibly through activation of T helper cells [96]. Direct stimulation of mouse and human dendritic cells by C5a activates them as demonstrated by increased expression of MHC class II, upregulation of co-stimulatory molecules such as CD80, CD86, CD40 and CD54, and secretion of cytokines of Th1 phenotype [97, 98]. C5a-mediated stimulation of DCs also decreased intracellular production of cAMP, which is a negative regulator of DC activation and function [98]. Furthermore, C5a interaction with C5aR activates PI3K, ERK1/2, NF-kB signaling pathways in DCs, which positively regulate antigen uptake, presentation and secretion of proinflammatory cytokines by DCs [97,98]. C5a was also shown to exhibit both synergistic as well as

antagonistic crosstalk with toll like receptors (TLRs) in functional modulation of DCs [97,98]. C5a decreased TLR4 mediated IL-12 secretion by DCs, but significantly increased secretion of other Th1 cytokines and CXC16 involved in NK cell activation.

C5a-C5aR interactions were shown also to be essential for generation of protective anti-viral CD8+ T cells. In contrast, genetic ablation or pharmacologic blocking of C5aR signaling predominantly generated of Th2 and regulatory T cell responses [99]. Furthermore, previous studies found that blocking C5a-C5aR interactions also impaired the ability of immune system to generate memory CD4+ T cells [100]. Recent evidences also found that DCs stimulated with recombinant C5a showed increased expression of multiple co-stimulatory molecules, migration to the draining lymph nodes and strong interaction with antigen-specific naïve T cells.

Besides immunostimulatory properties that promote antigen presentation and functional immune responses, C5a also possesses highly inflammatory properties. C5a is known to be the most potent mediator of local and systemic inflammatory responses and causes multiple immunological effects such as activating and recruiting neutrophils, induce spasmogenesis, increase vascular

permeability and stimulate the release of secondary inflammatory mediators from a variety of cell types (e.g., leukocytes and macrophages). Anaphylatoxin C5a is also a potent chemotactic agent and release at the site of the lesion C5a causes the release of histamine by the mast cells, the production of superoxide anions and the release of hydrolytic enzymes by the neutrophils and promotes the phagocytosis by the macrophages. By increasing the release of toxic proteins and oxygenated reagents, C5a also activates eosinophils. Moreover, the stimulation of macrophages by C5a results in the secretion of interleukin 1 (101).

1.4.4.3.2 Development of EP54 and EP67

C5a consists of highly ordered helical N-terminus (1-63) responsible for recognition and binding of C5aR and highly flexible disordered C-terminus (64-74) that exists as a finger-like projection from N-terminus (**Figure 1.2**) core responsible for transducing biological signal [102]. Decapeptides possessing the essential conformational features of C-terminus region of C5a showed full agonist activity compared to natural C5a. Conformationally biased, response-selective agonist of C5aR, EP54 (YSFKDMPLaR) was synthesized by amino acid residue modification of C-terminus region of C5a (65-74). EP54 retained C5a-like immunostimulatory properties as demonstrated by 5% of C5a-like induction of smooth muscle contraction but was devoid of C5a-like inflammatory properties as demonstrated

by only 0.1% of C5a's ability to induce mast cell degranulation. The unique structural and conformational features were shown to be well accommodated by C5aRs on antigen presenting cells such as DCs and macrophages, but not by C5aRs expressed on neutrophils. Furthermore, these conformational features increase stability of EP54 from proteolytic degradation by serum carboxy-peptidases. Additional modification of EP54 by introducing N-methylation generated EP67, which increased response-selectivity towards C5aR and reduced activity towards neutrophils up to 3000-fold compared to EP54 [103].

1.4.4.3.3 Vaccine Adjuvant Properties of EP54 and EP67

EP54 and EP67 were successfully used as adjuvants to generate antigen-specific humoral and cellular immune responses. Mice immunized with EP54 containing MUC1-constructs generated antibodies with class switched isotype, which were able to bind to MUC1- expressing cancer cell lines. EP54 and MUC1-CTL epitope containing vaccines also generated functional antigen specific cytotoxic T cell responses [102]. Mice and rats immunized with EP67-conjugated protein vaccines generated Th1-type humoral immune responses. Older mice immunized with EP67 conjugated to a model protein antigen ovalbumin were also able to generate higher antibody responses and Th1-type isotype switching which were not observed when a toll like receptor (TLR) ligand CpG or alum were used

as adjuvants [104]. The vaccine adjuvant properties of EP54 and EP67 in generating protective adaptive immune responses were shown demonstrated in various animal species (**Table 1.2**). EP54 and EP67 have shown to increase antigen processing and presentation capacity, and induced release of Th1 type cytokines by C5aR-bearing APCs [105].

Furthermore, EP67 was shown to reduces Methicillin-Resistant Staphylococcus aureus (MRSA) skin lesions. Subcutaneous administration of EP67 (250 µg), but not inactive scrambled EP67 or PBS vehicle, significantly reduced lesion size and recoverable CFU counts in outbred CD1 mice with dermal infections of MRSA (TCH1516 USA 300). These host-directed anti-MRSA effects are C5aR/CD88-dependent since they were not seen in C5aR knockout mice (CD88-/-). EP67 induced the recruitment of essential neutrophils and macrophages to the lesion site through cytokine/chemokine release from C5aR-bearing APCs in the lesion zone [106].

Moreover, administration of EP67 (200 µg) in the area of a MRSA (USA 300 LAC) biofilm infection around an implanted subcutaneous catheter resulted in a significant reduction of recoverable CFU counts within the biofilm and surrounding tissue. This effect was seen within 24 hrs of administration of EP67 and was sustained for up to 5 days post-administration. It was also shown that EP67

increased the recruitment of macrophages to the biofilm infection and induced the release of IL-12p40, CCL5, IL-17, IL-1β, and INFγ in the biofilm. These effects were not seen by the administration of inactive scrambled EP67 or vehicle (PBS) [107].

Furthermore, pulmonary delivery of EP67 was also found to protects against sub-lethal and lethal Infections of Influenza A. Intranasal insufflation of EP67 (25 µg in 30 µL) 24 hrs prior to influenza infection significantly reduced loss of body weight upon infection relative to mice that received no EP67 or vehicle (PBS) only. Likewise, infected mice that received EP67 (25 µg in 30 µL) 24 hrs post-infection showed a significant reduction in the loss of body weight relative to mice that received no EP67 or PBS only. This pulmonary administration of EP67 resulted in a significant influx of neutrophils, alveolar macrophages, dendritic cells, and natural killer cells in the bronchoalveolar lavage (BAL) fluids of both uninfected and infected mice. BALs from mice treated with EP67 also showed significantly higher amounts of IL-6, GM-CSF, KC (the murine equivalent of IL-8), IL1β, IL12p70, and MIP-1. These cellular and cytokine/chemokine influxes are consistent with the prophylactic and therapeutic effects of EP67 described above as well as the ability of EP67 to overcome the inherent immune suppressive effects of influenza infection. These effects were not observed in mice administered scrambled EP67

of PBS vehicle. Finally, intranasal insufflation of EP67 (240 µg in 30 µL) 24 hrs after administration of a lethal dose of influenza protected all mice (100% survival) up to 30 days. All untreated mice were dead 11 days post-infection [108]. The results summarized above underscore the capacity of the topochemical features expressed in EP67 to be selectively accommodated by C5aRs expressed on APCs vs. C5aRs expressed on neutrophils and, consequently, the ability to selectively activate C5aR-bearing APCs for the induction of host innate immunity, which reduces normal/resistant bacterial and viral infections. Thus, EP67 can be used as a template from which can be generated EP67 analogs even more effective in C5aR-bearing APC engagement and activation. The availability of such analogues would usher in the therapeutic concept of host-directed immunotherapy.

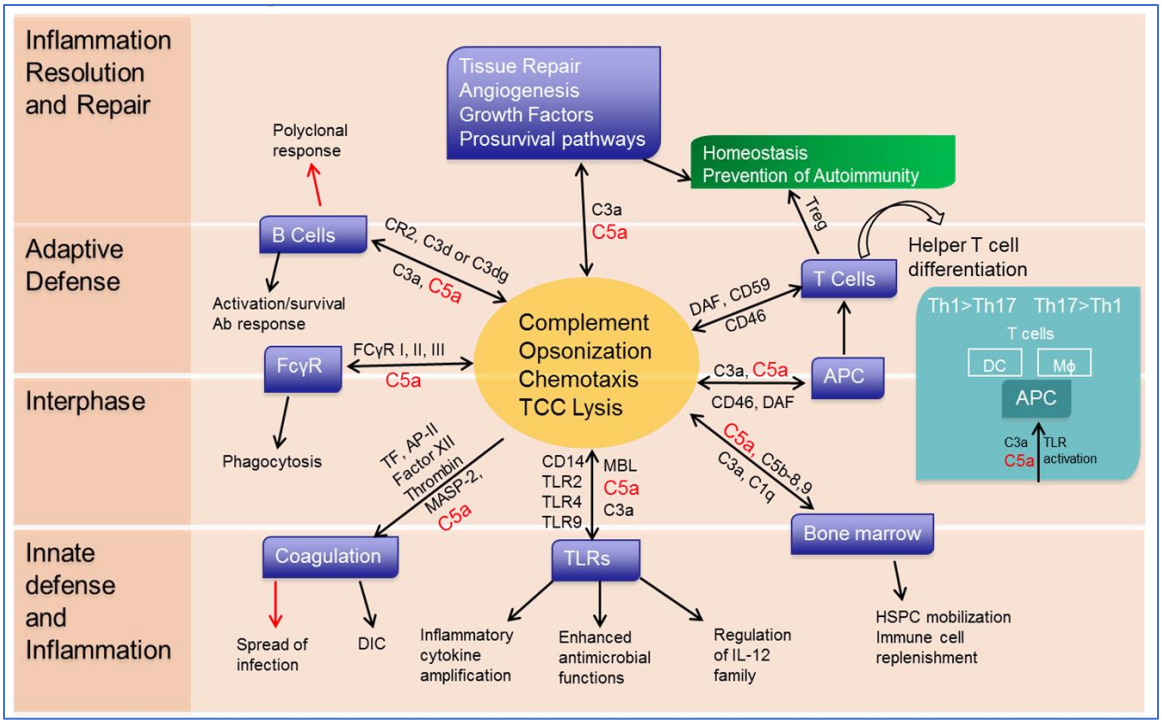


Figure 1.1 Role of Complement C5a in host defense and homeostasis [Adapted from 95].

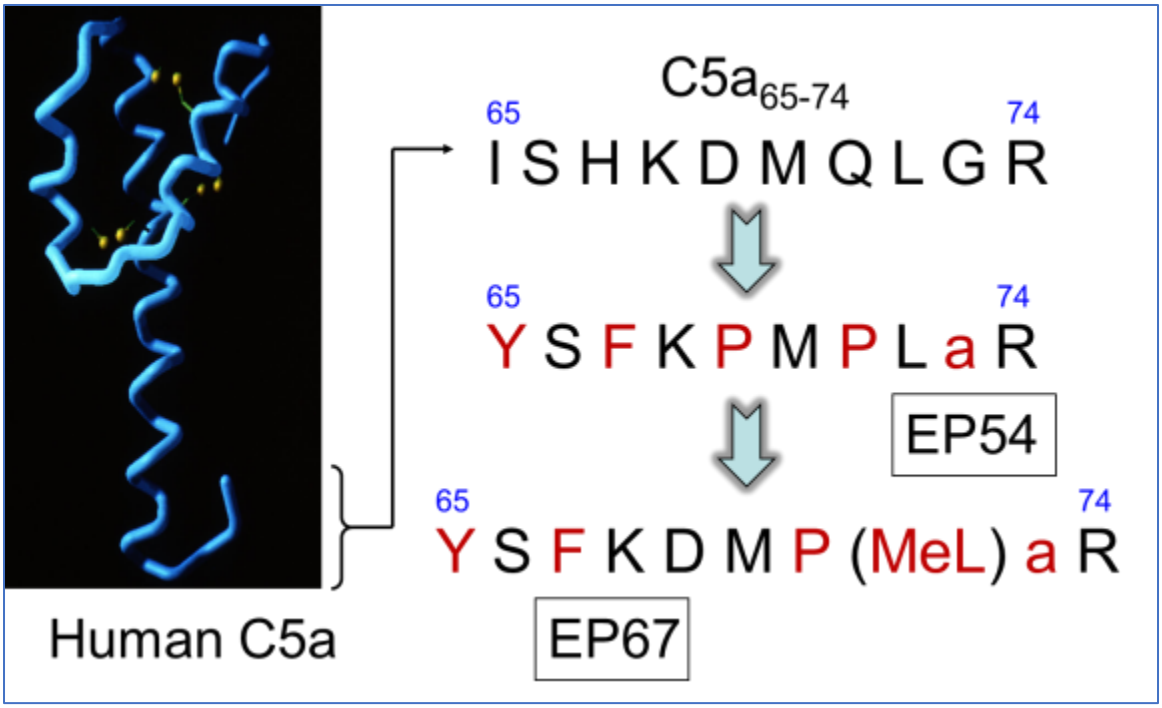


Figure 1.2 Structure of human C5a

Immunostimulants	Origin	Mechanism of action	Clinically application use
Microbially-derived immunostimulants			
BCG	Live mycobacteria	Stimulates macrophages	Bladder cancer, TB
Picibanil	Group A streptococcus pyrogenes	Stimulates natural killer cells, macrophages and lymphocytes	Lymphangiomas
Krestin	Fungal Polysaccharide	Stimulates CD8+ T cells and natural killer cells	Gastric and other cancers

Lentinan	Fungal Polysaccharide	Stimulates macrophages	Gastric cancer
Biostim	Klebsiella pneumoniae extraction	Stimulates macrophages	Chronic/recurrent infections
OM-85 (Broncho-Vaxom)	Lysate of eight bacterial pathogens	Stimulates macrophages	Chronic/recurrent infections
Pneumovax 23	Pneumococcal polysaccharides	Induce and activates B-cell-dependent immune response	Chronic/recurrent infections
Menactra	Meningococcal (Groups A, C, Y and W-135) Polysaccharide	Activates B and T cells	Chronic/recurrent infections
Thymus-derived immunostimulants			
Thymostimulin	Thymic peptide extract	Stimulates T cell maturation	Cancers and infections
Thym-uvocal	Thymic peptide extract	Stimulates T cell maturation	Cancer and AIDS

Thymomodulin	Thymic peptide extract	Stimulates T cell maturation	Infections, allergies and malignancies
Exogenous Cytokines immunostimulants			
filgrastim	Recombinant granulocyte colony-stimulating factor (G-CSF)	Stimulate bone marrow	Neutropenia
Interferon β-1a	Recombinant Interferon β-1a	Reduce neuron inflammation, increase the production of nerve growth factor and improve neuronal survival	Multiple sclerosis (MS)
Interferon γ-1b	Recombinant Interferon γ-1a		chronic granulomatous disease (CGD)

Interferon α-n3	Recombinant Interferon α-n3	upregulates the expression of MHC I proteins, enhances the activation of CD8+ T cells, and stimulate macrophages	Cancers and infections
Aldesleukin	Recombinant IL-2	Activates T cells, natural killer cells	Metastatic renal cell carcinoma and metastatic melanoma
Oprelvekin	Recombinant IL-11	Stimulate the bone marrow	Myeloid malignancies

Chemically modified immunostimulants			
Romurtide	Muramyl dipeptide	stimulates macrophages, increased production of neutrophils	chemotherapy-induced leukopenia, bone marrow recovery
Thymopentin	Pentapeptide (Arg–Lys–Asp–Val–Tyr)	Stimulate T cells	Cancers, rheumatoid arthritis and infections
Inosine pranobex	Salt of acetamidobenzoic acid and dimethylaminoisopropanol	Stimulate T cells	Infections and cancers

Table 1.1 Clinically licensed immunostimulants (Adapted from [2])

Vaccine Construct	Route	Immune Response	Ref
Peptide epitope from MUC1 glycoprotein conjugated to EP54	i.p.	↑IgG2b, IgG2c and IgM Abs	[102]
CTL peptide epitope derived from Hepatitis B surface Antigen (HBsAg) conjugated to EP54	s.c	Ag-specific CD8+ CTL responses against murine P815S target cells	[109]
Nicotine hapten conjugated to EP54	i.p.	Nicotine-specific Abs	[110]
Methamphetamine (meth) hapten conjugated to EP54	s.c /i.p.	Meth-specific Abs in sera	[111]
OVA conjugated to EP67	i.p.	OVA-specific Th1-like Ab class switch and OVA-specific proliferative responses in splenocytes	[112]
OVA conjugated to EP67	i.p.	↑ Ag-specific humoral	[113]
rPrp1,a protein from cell wall of *coccidioides* conjugated to EP67	i.p.	↑ humoral responses compared to alum and CpG	[113]
Peptide epitope derived from gp70 glycoprotein conjugated to EP54 and EP67	s.c	↑ CTL responses and ↓RAW117-H10 growth	[114]

Live spores of attenuated vaccine strain of *Coccidioides posadasii* conjugated to EP67	s.c	↑ IgG1, IgG2a, Th1, and Th17 immune responses	[115]
CTL peptide epitopes from MCMV PP89 and M84 conjugated to EP67	i.n.	↑ CTL responses ↓ MCMV titers	[116]

Table 1.2 Vaccine adjuvant properties of EP54 and EP67

CHAPTER 2

Hypothesis and Specific Aims

2.1 Research Objectives and Hypotheses

There is an unmet need for safe and efficacious approaches to treat infections through the stimulation of systemic and mucosal innate immunity with little toxicity. One approach is to stimulate innate immune responses with a safe and effective immune stimulatory molecule. Such “host-directed immunotherapy” will stimulate the body’s inherent first line of defense against infections while exerting few/no mutational pressure on the infecting pathogen. Our group previously has shown the potential of host-directed immunotherapy against viral infections in mice by the administration of our lead immune stimulatory candidate EP67**.**

The decapeptide EP67 is a novel host-derived adjuvant based on the C-terminal of C5a that selectively activates antigen presenting cells (APCs)-monocytes, macrophages, and dendritic cells over neutrophils. In contrast to C5a65-74 from the C5a parent molecule, EP67 has topographical features that are well accommodated by C5aRs expressed on (APCs), but not C5aRs neutrophils. Thus, EP67 induces host innate immune responses against normal and resistant infections via engagement of C5aR-bearing APCs with little/no inflammatory side-effects associated with the direct activation of neutrophils.

The Pro residue at position 7 (Pro7) is critical for the potency and selectivity of EP67. It was placed in this position to force an extended conformation at the carbonyl carbon between the ω and ψ bonds in the Met backbone to its N-terminus, a feature that was found to be biologically important from SAR studies with the previous analog of EP67, EP54. The Pro residue, however, undergoes cis/trans isomerization that leads to conformers of EP67 in solution, making it unclear whether cis/trans isomerization is important to the biological properties of EP67 or if one conformer is more effective than the other. What is clear is that the conformational influence of Pro at this position is important for the activity and bio-selectivity of EP67**.** ***Our long-term goal*** is to increase the potency and efficacy of EP67 to improve host-directed immunotherapy and vaccine applications. ***The overall objective of this proposal*** is to generate analogs of EP67 with increased potency, efficacy, and bio-selectivity. ***Our central hypothesis*** is that replacing Pro in position 7 with structurally diverse residues that are likely to shift the cis/trans conformer equilibrium or lock the conformation in either a cis or trans orientation increases the potency and APC-selectivity of EP67.

2.2 Specific Aims

Specific Aim 1. Synthesize a series of EP67 analogs with residue substitutions for Pro at position 7. As noted above, our goal is to generate a series of EP67 analogs to better understand the conformational influence of Pro in the Met-Pro-MeLeu triad region of EP67 and to increase the structural diversity within this biologically important core of EP67 with the objective of increasing the potency and bio-selectivity of this unique innate immune stimulatory decapeptide.

Specific Aim 2. Determine analog potency and bio-selectivity in C5aR-bearing human phagocytes (monocytes, monocytes-derived macrophages (M0-MDM), monocytes-derived immature DCs (MDDC), and C5aR-bearing human neutrophils. EP67 analogs generated in Aim 1 will be assessed for their potency to C5aRs on human monocytes, M0-MDM, MDDC and neutrophils, as measured by cytokine release (IL-1β, IL-6, and TNF-α) from monocytes, M0-MDM, MDDC and myeloperoxidase (MPO) release from neutrophils, and from these potency assays, their bio-selectivity relative to EP67 and natural C5a will be determined.

CHAPTER 3

MATERIALS AND METHODS

3.1 Peptide synthesis, purification, and characterizations

With the assistance of an AAPPTEC Apex 396 Synthesizer, all peptides were assembled on preloaded Fmoc-Arg(pbf)-Wang resin using Fmoc-amino acid derivatives and N-(dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU) in the presence of excess DIEA. Peptides were cleaved from the resin and freed of sidechain protecting groups by stirring the peptide-resin in a mixture of TFA (87.5%), phenol (5%), water (5%), and triisopropylsilane (2.5%) for 2h at room temperature [117,118]. All peptides were subjected to analytical reversed-phase HPLC employing three different columns under the same gradient elution conditions. The three columns were an ACE 5 C18-300 column (250 x 4.6 mm, 5 µm, 300 Å pore size, catalog number ACE-221-2546), ACE 5 Phenyl-300 column (250 x 4.6 mm, 5 µm, 300 Å pore size, catalog number ACE-225-2546) and ACE 5 CN-300 column (250 x 4.6 mm, 5 µm, 300 Å pore size, catalog number. ACE-224-2546) from MAC-MOD Analytical Inc. (Chadds Ford, PA). Solvent A was water containing 0.1% trifluoroacetic acid (TFA, v/v) and solvent B was a mixture of acetonitrile and water (3/2, v/v) containing 0.1 % TFA. Peptides were eluted from the columns by increasing the percentage of solvent B from 5 to 100% over 50 minutes and column effluent was continuously monitored at 214 nm. Peptide

masses were obtained from the Mass Spectrometry and Proteomics Core Facility at the University of Nebraska Medical Center. Masses were measured on an Orbitrap Fusion Lumos from ThermoScientific (Waltham MA) and with ≤ 0.5 ppm error. Amino acid compositions of all peptides were provided by the Protein Structure Core Facility at the University of Nebraska Medical Center. Samples were subjected to vapor-phase hydrolysis in constant boiling 6M hydrochloric acid for 24 hours prior to being loaded on to a Hitachi 8800 Amino Acid Analyzer.

3.2 De novo peptide structures

PEP-FOLD was used to generate the initial conformation of $C5a_{65-74}$. Analogs of $C5a_{65-74}$ were generated in YASARA and refined for 500 ps using the built-in md_refine macro. Each refined structure was then used in a 50 ns molecular dynamics (MD) simulation. All molecular dynamics simulations and post-analysis used Desmond as bundled with the Schrodinger software suite. Each peptide was placed in a cubic box with periodic boundaries. No dimension of the box was allowed closer than 12 Angstroms to allow the peptides room to unfold. The box was filled with TIP4P water and neutralized by adding the appropriate Na+ or Cl- ions. Salt concentration in the box was set to 0.05 M NaCl. All simulations first used Schrodinger's built in relaxation protocol before the main MD run. The main 50 ns MD run was an NPT ensemble with temperature at 298K and pressure

at 1 atm. Noose-Hoover chain and Martyna-Tobias-Klein were the thermostat and barostat methods, respectively. The average structure of the major cluster of each trajectory was then extracted for comparison in YASARA [119,120].

3.3 Isolation of human monocytes and neutrophils from human whole blood

Fresh whole blood [1 unit = 450 mL] was drawn from human healthy male donors (aged 19–40 years) into vacutainer bags (Research Innovative, USA) containing EDTA. $CD14^+$ monocytes [165 mL of blood/donor] were isolated by MACS® technology using CD14 MicroBeads (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Isolated monocytes [$1x10^6$ cells/well] were cultured into 24-well plates, and allowed to adhere [2 hrs] in complete culture medium (CCM) (RPMI 1640, 2 mM L-glutamine, 1% autologous plasma, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1x vitamins, 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate (Invitrogen, Carlsbad, CA) [1 mL/well] and incubated at 37 °C and 5% CO_2. Non-adherent cells were discarded, and the adherent cells were washed carefully twice with sterile D-PBS ("PBS" without Ca^{2+} or Mg^{2+}, GE Healthcare Life Sciences: SH30028.02) [1 mL] [120,121].

Neutrophils were also isolated from same donors [10 mL of blood/donor] using the MACSxpress® Whole Blood Neutrophil Isolation Kit, a MACSmix™ Tube Rotator, and a MACSxpress Separator (Miltenyi Biotec, Germany) according to the

manufacturer's protocol. Isolated neutrophils [1x10^6 cells/well] were cultured into 24-well plates in CCM [1 mL] and incubated at 37 °C and 5% CO_2. Non-adherent cells were discarded, and the adherent cells were washed carefully twice with sterile D-PBS [123]. The purity of the isolated monocyte and neutrophil was determined by labeling cells with CD14- PE-Vio615, CD-15APC, and CD-16PE human antibodies (Miltenyi Biotec, Germany) before and after separation, and analyzed by flow cytometry. Briefly, isolated cells [1x10^6] were resuspended in cell staining buffer (BD Biosciences) [100 μL] and indicated antibodies [5 μL/each antibody]. Cells were mixed and incubated in the dark in the refrigerator [2−8 °C, 10 min.]. Then, cells were washed by adding staining buffer [1 mL] and pelleted [300 RCF, RT, 10 min.]. The supernatant was completely aspirated, and cell pellets were resuspended in FACS staining buffer [0.5 mL] for analysis by cytometry. Cells were analyzed on a BD LSR II flow cytometer (Becton and Dickinson, La Jolla, CA) with BD High Throughput Sampler. Flow cytometer was compensated using single stained cells, maximum number of events were acquired and analyzed by FlowJo software (Tree Star, Ashland, OR, USA) [124,125].

3.4 Generate unpolarized human monocyte-derived macrophages (M0-MDM), and human monocyte-derived dendritic cells (MDDC)

Generation of unpolarized human monocyte-derived macrophages (M0-MDM) was done by culturing monocytes [1×10^6 cells/well] in CCM [1 mL] containing recombinant human M-CSF (Miltenyi Biotec) [50 ng/mL] in 24 well plates. On day 3, half media [0.5 mL] from each well was carefully removed and replenished with CCM containing rhM-CSF. After the differentiation, the cell supernatants were completely removed by gentle aspiration and cells were washed once with warm sterile PBS [126-128].

To generate human monocytes-derived immature dendritic cells (MDDC), isolated monocytes [1×10^6 cells/well] were cultured in CCM [1 mL] supplemented with recombinant human IL-4 [50 ng/mL] and recombinant human GM-CSF [160 ng/mL] (Miltenyi Biotec) in 24-well culture dishes. On days 3 and 6, half of the volume [0.5 mL] was gently aspirated from each well and replenished with CCM containing GM-CSF and IL-4 [128-130]. The morphology of differentiated cells (macrophages as well as immature Mo-DCs) were analyzed by placing cells in a 24-well plate and allowed to sediment. Images of the cells were captured using a light microscope with phase-contrast at a 400× magnification.

3.5 Determine the potency of EP67 analogs

Analog potency was determined by measuring the half-maximal effective concentration (EC_{50}). EC_{50} values for each analog were determined in two separate assays: cytokine release from human mononuclear phagocytes (human monocytes, M0-MDM, and MDDC) and myeloperoxidase (MPO) release from human neutrophils. Full dose-response curves were generated in each experiment, and the EC_{50} values were determined. Briefly, monocytes, M0-MDM, and MDDC [$1x10^6$ cells/well] were incubated in the presence of serial concentrations of C5a desArg, EP54, EP67, and EP67 analogs in standard cell culture conditions for 24 hrs. Supernatants were collected and assayed for the presence and amount of the IL-1β, IL-6, and TNF-α (BioLegend, USA) using standard ELISA methods [102,131].

In human neutrophils, the release of the proteolytic enzyme MPO was measured. Briefly, neutrophils were incubated in the presence of serial concentrations of C5a desArg, EP54, EP67, and EP67 analogs in standard cell culture conditions for 24 hrs. Supernatants were collected and assayed for the presence and amounts of MPO using standard ELISA [103,131].

3.6 Determine the bio-selectivity of EP67 analogs

Analog selectivity for cytokine release (IL-6, and TNF-α) from phagocytes cells (monocytes, M0-MDM, and MDDC) vs. MPO release from neutrophils were determined by the following equation:

$$\text{Selectivity} = \text{antilog}[(-\Delta\text{phagocytes}) - (-\Delta\text{neut})]$$

, where Δ is the log potency ratio (pD_2 C5a – pD_2 analog) and $pD_2 = -\log EC_{50}$. The "selectivity" of natural C5a desArg were set at value of 1 using this equation since it is equipotent in both cytokine release from monocytes and MPO release from neutrophils. Thus, differences in the potencies between these two C5aR-bearing cells can be assessed relative to C5a desArg; i.e., the greater the value from the above equation, the greater the selectivity relative to C5a desArg. This is the pharmacologically accepted means of determining selectivity between two compounds and was used by us to determine the selectivity of EP67 for engagement and activation of C5aR-bearing phagocytes over that of C5aR-bearing neutrophils (131).

3.7 Compare the IL-6 and TNF-α cytokine release rates from human monocytes, M0-monocyte-derived macrophages, and immature monocyte-derived dendritic cells.

Human monocytes (MC), M0-monocyte-derived macrophages (M0-MDM), and immature monocyte-derived dendritic cells (MDDC) were prepared from the whole blood of healthy, young human adult male donors. Cell types were treated with the calculated EC_{50} of human C5a desArg pooled from the blood of multiple donors (black closed circles), 1st-generation EP54 (black closed squares), 2nd-generation EP67 (black closed triangles), [Cha^{7}]EP67 (red open circles), [Leu^{8}]EP67 (green open squares), or [Cha^{7}, Leu^{8}]EP67 (blue open triangles). At the indicated time points, average concentrations of IL-6 or TNF-α released into cell culture media by human monocytes, M0-MDM, and MDDC were determined by ELISA.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Replacing Pro^7 with cyclohexylalanine and/or *N*-methyl Leu^8 with Leu selectively affects the potency and efficacy of EP67 in human mononuclear phagocytes

We originally proposed that Pro^{7}nme-Leu^8 increases the potency and selective activation of mononuclear phagocytes vs. neutrophils vs. EP54 by extending the peptide backbone. Thus, we wanted to identify amino acid substitutions that would eliminate cis/trans isomerization of EP67 while still extending the peptide backbone. We hypothesized that substituting an amino acid that eliminates cis/trans isomerization and has a bulky side chain such as cyclohexylalanine (Cha) will extend the peptide backbone of EP67 to a similar extent as Pro^{7}nme-Leu^8 without adversely affect potency and efficacy. RMSD traces from d*e novo* peptide structure prediction showed long periods of low RMSD to the major cluster, suggesting C5a$_{65\text{-}74}$ maintains an alpha-helical backbone conformation throughout most of the simulation (**Figure 4.1**). In contrast, although [Cha^{7}Leu^8] EP67 is masked in the major cluster view, RMSD traces indicate that amino acid substitutions introduced to EP67 and EP67 analogs greatly increase the flexibility of C5a$_{65\text{-}74}$. This increased flexibility is more similar to the disordered structure of C5a$_{65\text{-}74}$ observed by MMR when part of native C5a, although a short helix was observed. The similarity between C5a$_{65\text{-}74}$ and [Leu^8] EP67 RMSD traces

suggests [Leu^{8}] EP67 best preserves the predicted alpha-helical backbone of C5a$_{65-74}$. We hypothesized that nme would not be required to extend the peptide backbone with Cha

To determine if replacing Pro^{7} and/or *N*-methyl Leu^{8} with amino acids that induce similar structural changes will affect the potency and efficacy of EP67 in human mononuclear phagocytes, we first replaced Pro^{7} with Cha ([Cha^{7}]EP67), nme-Leu^{8} with native Leu ([Leu^{8}]EP67), or Pro^{7} and nme-Leu^{8} with Cha and native Leu, respectively ([Cha^{7}Leu^{8}]EP67) (**Table 4.1**), determined purity (**Table 4.2**) (**Figure 4.2**) and molecular mass (**Table 4.3**) (**Figure4.3**), and confirmed the amino acid sequence (**Table 4.4**) of each peptide. We then prepared human CD14^{+} / CD14^{+}CD16^{+} monocytes (MC), unpolarized human monocyte-derived macrophages (M0-MDM), and human monocyte-derived dendritic cells (MDDC) from the whole blood of healthy, human adult male donors (**Figure 4.4**) and compared dose-dependent secretion of IL-1β, IL-6, and TNF-α after treating with pooled human C5a desArg (parent molecule), EP54 (1st generation ADI), EP67 (2nd-generation ADI), or EP67 analogs for 24 h (**Figure 4.5**). We used “classical” CD14^{HI} MC because they represent 85% to 95% of human monocytes and will include the “intermediate subset” CD14+CD16+ MC and potentially the “non-classical” CD14^{LO}CD16^{HI} MC [1], unpolarized M0-MDM (Day 6 MCF) because they

are similar to tissue resident macrophages from healthy tissues, and MDDC (Day 7 GM-CSF/IL-4). Mononuclear phagocytes (monocytes, macrophages, and dendritic cells) are critical immune sensors in the body that play a key role in initiating and guiding innate and adaptive immunity. Acute inflammation augments innate and adaptive immune responses and is driven by the secretion of three major proinflammatory cytokines: IL-1β, IL-6, and TNF-α. We compared dose-dependent secretion of IL-1β, IL-6, and TNF-α because they are the major early pro-inflammatory cytokines that drive acute inflammation in support of innate and adaptive immune responses and are, consequently, good surrogate markers for the activation of mononuclear phagocytes.

Sigmoidal dose-responses curves were observed for IL-1β secretion from MC, M0-MDM, and MDDC over the concentration range of C5a desArg (**Figure 4.6**), whereas IL-1β was not detected after treatment with up to 1 mM EP54, EP67, or EP67 analogs (not shown). In contrast to IL-1β, sigmoidal dose-response curves were observed for IL-6 and TNF-α secretion from MC, M0-MDM, and MDDC over the concentration ranges of C5a desArg (black circles), EP54 (black squares), EP67 (black triangles), and EP67 analogs (colored open symbols) (**Figure 4.5**). This suggests the current ADI do not stimulate the secretion of one or more

cytokines and, possibly, chemokines from human mononuclear phagocytes that are normally stimulated by the C5a/C5a desArg parent molecule.

The EC_{50} for EP67 (**Figure 4.7 A**, black triangles) ranged from 14.6- (TNF-α from MC) to 89.5-fold greater (IL-6 from MDDC) than C5a desArg (**Figure 4.7 A**, black circles) (**Table 4.5**). Furthermore, the EC_{50} for EP67 (**Figure 4.7 A**, black triangles) was similar to the EC_{50} for EP54 (**Figure 4.7 A**, black squares), with the exception of TNF-α secretion from MC (35% less) (121 ± [-0.2,+0.3] [95% CI] vs. 186 [-0.2,+0.3] nM) and IL-6 secretion from M0-MDM (47% less) (167 ± [-0.3,+0.3] [95% CI] vs. 317 [-0.9,+1] nM) (**Table 4.5**). The E_{MAX} for EP54 ranged from 24 to 47% lower (**Figure 4.7 B**, black squares) than pooled human C5a desArg (**Figure 4.7 B**, black circles), whereas and the E_{MAX} for EP67 (**Figure 4.7 B**, black triangles) ranged from 6 to 27% lower depending on the cytokine and mononuclear phagocyte (**Table 4.5**). Thus, EP54 and EP67 are both generally partial agonists for activating human mononuclear phagocytes. The E_{MAX} for EP67 (**Figure 4.7 B**, black triangles) was 23.6% to 34% greater than the E_{MAX} for EP54 (**Figure 4.7 B**, black squares) also depending on the cytokine and mononuclear phagocyte. Thus, the potency of EP67 is similar or greater than EP54 depending on the secreted cytokine and human mononuclear phagocyte, whereas the efficacy of EP67 for IL-6 and TNF-α secretion is greater than EP54 in human mononuclear phagocytes.

[Cha^{7}]EP67 (**Figure 4.7 A**, red open circles) did not affect the EC_{50} of EP67 **(Figure 4.7 A**, black triangles) for IL-6 and TNF-α from MC, decreased EC_{50} for IL-6 (29%) but not TNF-α from M0-MDM, and did not affect the EC_{50} for IL-6 and TNF-α from MDDC. In contrast, [Leu^{8}]EP67 (**Figure 4.7 A**, green open triangles) did not affect the EC_{50} for IL-6 and TNF-α from MC, M0-MDM, or MDDC. In contrast, [Cha^{7}Leu^{8}]EP67 (**Figure 4.7 A**, blue open triangles) did not affect the EC_{50} for IL-6 and TNF-α from MC, decreased the EC_{50} for IL-6 (31%) without affecting the EC_{50} for TNF-α from M0-MDM, and did not affect the EC_{50} for IL-6 but decreased the EC_{50} for TNF-α (39%) from MDDC. Thus, [Cha^{7}]EP67 selectively increases potency in M0-MDM without affecting potency in MC or MDDC, [Leu^{8}]EP67does not affect potency in MC, M0-MDM, or MDDC, and [Cha^{7}, Leu^{8}] selectively increases potency in M0-MDM and MDDC without affecting potency in MC compared to EP67.

[Cha^{7}]EP67 (**Figure 4.7 B**, red open circles) did not affect E_{MAX} of EP67 (**Figure 4.7 B**, black triangles) for IL-6 but increased E_{MAX} for TNF-α (9.5%) from MC and decreased E_{MAX} for IL-6 and TNF-α from M0-MDM (9.5%, 16%) and MDDC (5.2%, 16.6%). [Leu^{8}]EP67 (**Figure 4.7 B,** green open squares) decreased E_{MAX} for IL-6 (9.3%) without affecting E_{MAX} for TNF-α from MC, did not affect E_{MAX} for IL-6 but decreased E_{MAX} for TNF-α (23%) from M0-MDM, and, unexpectedly,

greatly increased E_{MAX} for IL-6 and TNF-α from MDDC (41%, 45%) to levels similar (IL-6) or greater (TNF-α) than C5a desArg (**Figure 4.7 B**, black circles). In contrast, [Cha^{7}Leu^8]EP67 (**Figure 4.7 B**, blue open triangles) did not affect E_{MAX} for IL-6 and TNF-α from MC, but decreased E_{MAX} for IL-6 and TNF-α from M0-MDM (21%, 21%) and MDDC (16%, 10%), respectively. Thus, [Cha^7]EP67 selectively increases efficacy in M0-MDM and MDDC, [Leu^8]EP67 selectively decreased efficacy in MC and M0-MDM but significantly increases efficacy in MDDC, whereas and [Cha^7, Leu^8]EP67 does not affect efficacy in MC but decreases in M0-MDM and MDDC compared toEP67.

As a whole, these results indicate that, compared to EP67, (i.) [Cha^7]EP67 does not affect potency but selectively increases efficacy in MC, selectively increases potency but decreases efficacy in M0-MDM, and does not affect potency but decreases efficacy in MDDC (ii.) [Leu^8]EP67 does not affect potency in MC, M0-MDM, or MDDC but selectively decreases efficacy in MC and M0-MDM and greatly increases efficacy in MDDC and (iii.) [Cha^{7}Leu^8]EP67 does not affect potency or efficacy in MC, selectively increases potency but decreases efficacy in M0-MDM, and selectively increases potency but decreases efficacy in MDDC. Thus, replacing Pro^7 with cyclohexylalanine and/or N-methyl Leu^8 with Leu affects

the potency and efficacy of EP67 depending on the human mononuclear phagocyte and secreted cytokine.

4.2 Replacing Pro^7 with Cha and/or nme-Leu^8 with Leu does not affect EP67 potency in human neutrophils but selectively decreases efficacy

To determine if replacing Pro^7 with Cha and/or nme-Leu^8 with Leu affects the potency and efficacy of EP67 in human neutrophils (NP), we isolated neutrophils from the whole blood of healthy, young human adult male donors by using MACSxpress® Whole Blood Neutrophil Isolation Kit, a MACSmix™ Tube Rotator, and a MACSxpress Separator (Miltenyi Biotec, Germany) (**Figure 4.4**) then compared dose-dependent secretion of myeloperoxidase (MPO) from NP after treating with pooled human C5a desArg, EP54, EP67, or EP67 analogs for 24 h (**Figure 4.8**). Sigmoidal dose-responses curves were observed for MPO secretion from NP over the concentration ranges of C5a desArg, EP54, EP67, and EP67 analogs (**Figure 4.8 A**).

The EC_{50} for EP54 (**Figure 4.8 B**, black squares) was 3000-fold greater than C5a desArg (7 ± 1 nM [95% CI] vs. 21 [-3,+4] µM) in NP, whereas the EC_{50} for EP67 (**Figure 4.8 B**, black triangles) was 22,857-fold greater than C5a desArg (**Figure 4.8 B**, black circles) (7 ± 1 nM [95% CI] vs. 160 [-17,+20] µM) (**Table 4.6**). Furthermore, the E_{MAX} for EP54 (**Figure 4.8 C**, black squares) was 1.7-fold less

than C5a desArg (5.6 ± 0.2 [95% CI] vs. 9.3 ± 0.3 ng/mL), whereas the E_{MAX} for EP67 (**Figure 4.8 C**, black triangles) was 2.7-fold less than C5a desArg (3.5 ± 0.1 [95% CI] vs. 9.3 ± 0.3 ng/mL) (**Table 4.6**). The EC_{50} for EP67 was also 3-fold greater than EP54 (160 [-17,+20] [95% CI] vs. 21 [-3,+4] μM) and the E_{MAX} was 1.6-fold less than EP54 (3.5 ± 0.1 [95% CI] vs. 5.6 ± 0.2 ng/mL) (**Table 4.6**). Thus, consistent with previous studies (131), the potencies and efficacies of EP54 and EP67 are well below human C5a desArg and the potency and efficacy of EP67 is less than EP54 in human neutrophils.

None of the EP67 analogs (**Figure 4.8 B**, colored open symbols) affected the EC_{50} for MPO secretion from NP compared to EP67 (**Figure 4.8 B**, black triangle). In contrast, [Cha^7]EP67 (**Figure 4.8 C**, red open circle) and [Cha^7Leu^8]EP67 (**Figure 4.8 C**, blue open triangle) decreased E_{MAX} for MPO secretion by 21% and 24%, respectively (**Table 4.6**), whereas [Leu^8]EP67 (**Figure 4.8 C**, green open square) did not affect the E_{MAX} compare to EP67 (**Figure 4.8 C**, black triangle). Thus, replacing Pro^7 with cyclohexylalanine and/or N-methyl Leu^8 with Leu has no effect on the potency but selectively decreases the efficacy of EP67 in human neutrophils.

4.3 Replacing Pro^7 with Cha and/or nme-Leu^8 with Leu does not affect selective activation of human mononuclear phagocytes over human neutrophils

To determine if replacing Pro^7 with Cha and/or nme-Leu^8 with Leu affects the selective activation of human mononuclear phagocytes over human neutrophils, we compared the ability of EP54, EP67, or EP67 analogs vs. pooled human C5a desArg to selectively stimulate secretion of IL-6 or TNF-α from human monocytes (MC), unpolarized monocyte-derived macrophages (M0-MDM), and monocyte-derived dendritic cells (MDDC) over secretion of myeloperoxidase (MPO) from human neutrophils (NP) 24 h after treatment (**Figure 4.9**). Selectivities of EP67 (**Figure 4.9**, black triangles) for stimulating MC, M0-MDM, and MDDC over NP were 9- to 15-fold greater than EP54 (**Figure 4.9**, black squares) depending on cytokine and mononuclear phagocyte. EP67 selectivity for stimulating human mononuclear phagocytes over human neutrophils is greater than EP54.

None of the EP67 analogs (**Figure 4.9**, colored open symbols) affected selectivities for stimulating IL-6 or TNF-α secretion from MC (**Figure 4.9 A**), M0-MDM (**Figure 4.9 B**), or MDDC (**Figure 4.9 C**) over stimulating the secretion of MPO from NP compared to EP67 (**Figure 6C**, black triangles)

(**Table 4.7**). Thus, replacing Pro^{7} with cyclohexylalanine and/or N-methyl Leu^{8} with Leu does not affect the selective activation of human mononuclear phagocytes over human neutrophils by EP67.

4.4 Replacing Pro^{7} with Cha and/or nme-Leu^{8} from the structure of EP67 does not affect kinetics of cytokine release from human mononuclear phagocytes.

To next determine if replacing Pro^{7} with Cha and/or nme-Leu^{8} with Leu affects the kinetics of cytokine release from human mononuclear phagocytes, we compared the release of IL-6 and TNF-α from human monocytes, M0-MDM, and MDDC 24 and 48 h after treatment with the EC50 of [Cha7,Leu8]EP67, human C5a desArg, EP54, or EP67 (**Figure 4.10**). C5a desArg (black bars) had the highest levels of IL-6 and TNF-α release after 24 h, whereas [Cha7, Leu8] EP67 (red bars), EP54 (green bars), and EP67 (blue bars) had the highest levels after 48 h. Thus, replacing Pro^{7} with Cha and/or nme-Leu^{8} with Leu from the structure of EP67 does not affect the kinetics of cytokine release from human mononuclear phagocytes.

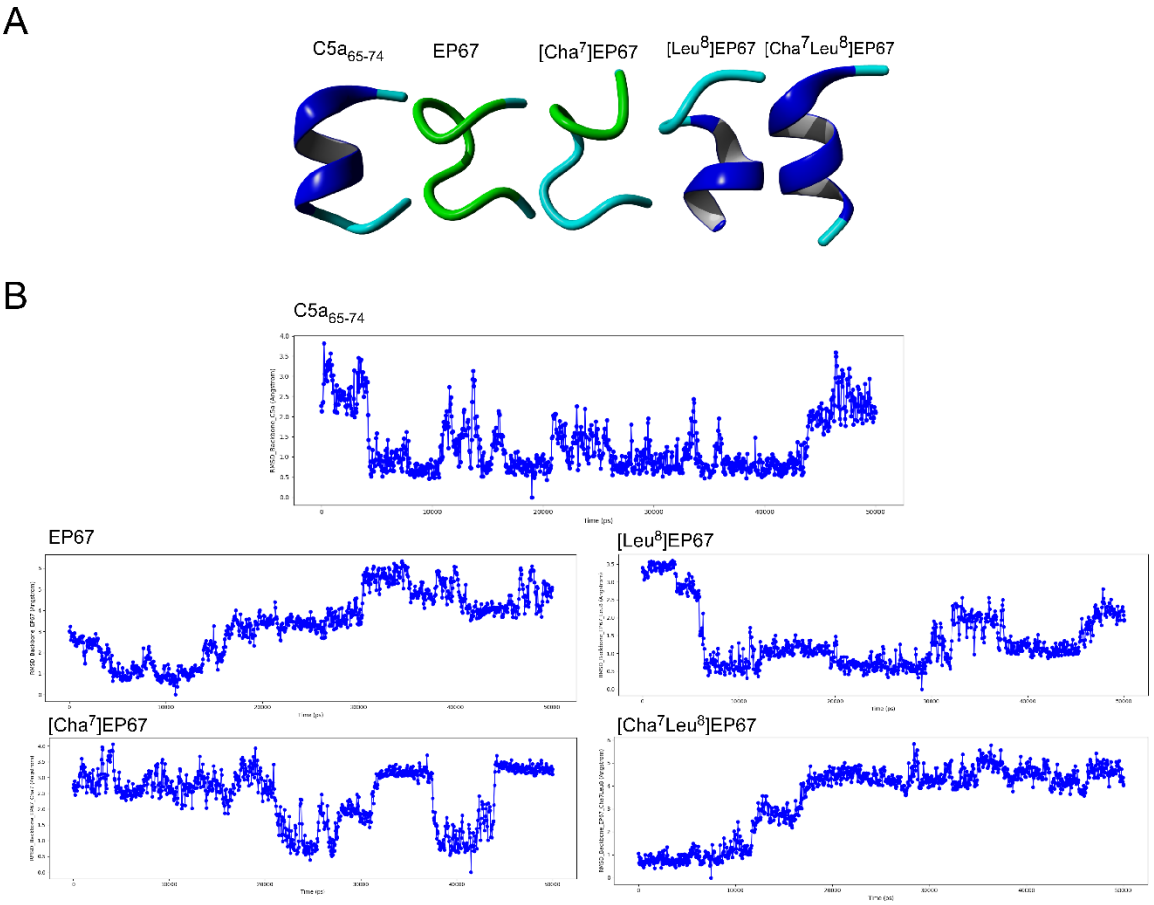


Figure 4.1 De novo structure of C5a desArg, EP54, EP67, and EP67 analogs. PEP-FOLD was used to generate the initial conformation of $C5a_{65-74}$. Analogs of $C5a_{65-74}$ were generated in YASARA and refined for 500 ps using the built-in md_refine macro. Each refined structure was then used in a 50 ns molecular dynamics (MD) simulation. All molecular dynamics simulations and post-analysis used Desmond as bundled with the Schrodinger software suite. Each peptide was placed in a cubic box with periodic boundaries. No dimension of the box was allowed closer than 12 Angstroms to allow the peptides room to unfold. The box was filled with TIP4P water and neutralized by adding the appropriate Na+ or Cl- ions. Salt concentration in the box was set to .05 M NaCl. All simulations first used Schrodinger's built in relaxation protocol before the main MD run. The main 50 ns MD run was an NPT ensemble with temperature at 298K and pressure at 1 atm. Noose-Hoover chain and Martyna-Tobias-Klein were the thermostat and barostat methods, respectively. The average structure of the major cluster of each trajectory was then extracted for comparison in YASARA.

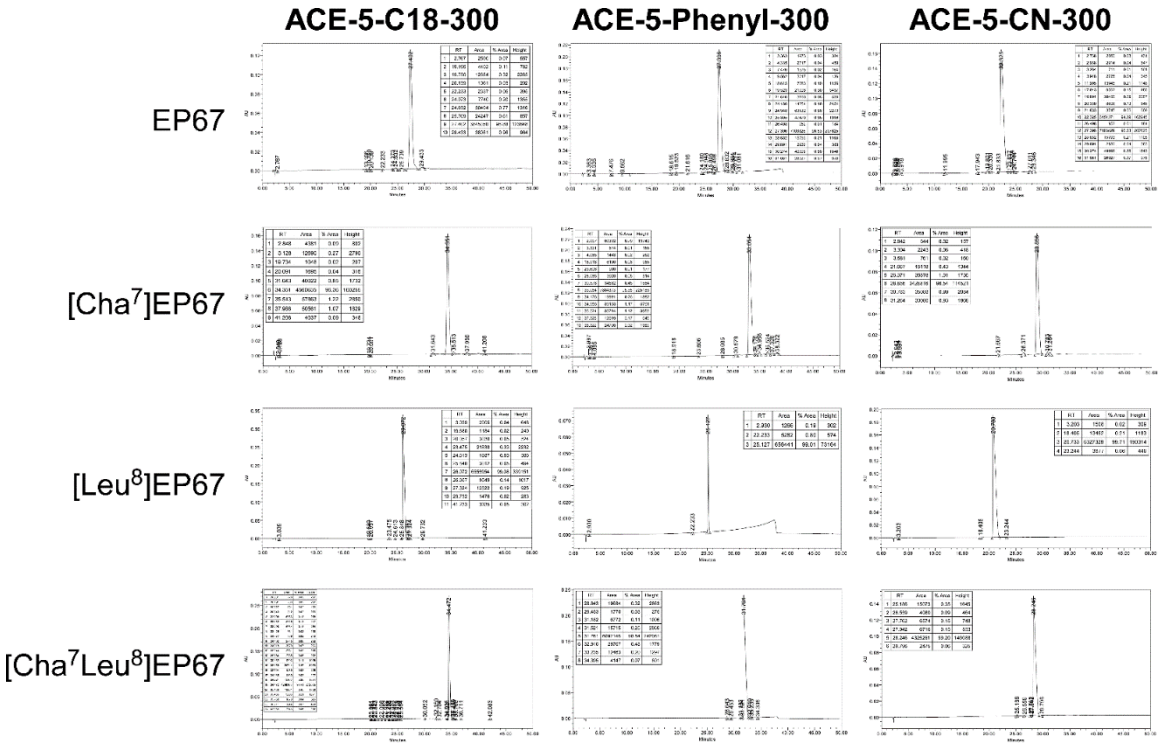


Figure 4.2 RP-HPLC chromatograms of EP67 and EP67 analogs.

Cha = cyclohexylalanine. Peptides were subjected to analytical reversed-phase HPLC employing three different columns under the same gradient elution conditions: an ACE 5 C18-300 column (250 x 4.6 mm, 5 µm, 300 Å pore size, catalog number ACE-221-2546), an ACE 5 Phenyl-300 column (250 x 4.6 mm, 5 µm, 300 Å pore size, catalog number ACE-225-2546), or an ACE 5 CN-300 column (250 x 4.6 mm, 5 µm, 300 Å pore size, catalog number. ACE-224-2546) from MAC-MOD Analytical Inc. (Chadds Ford, PA). Solvent A was ddH_2O containing 0.1% trifluoroacetic acid (TFA, v/v) and solvent B was a mixture of acetonitrile and ddH_2O (3/2, v/v) containing 0.1 % TFA. Peptides were eluted from the columns by increasing the percentage of solvent B from 5 to 100% over 50 minutes while column effluent was continuously monitored at 214 nm

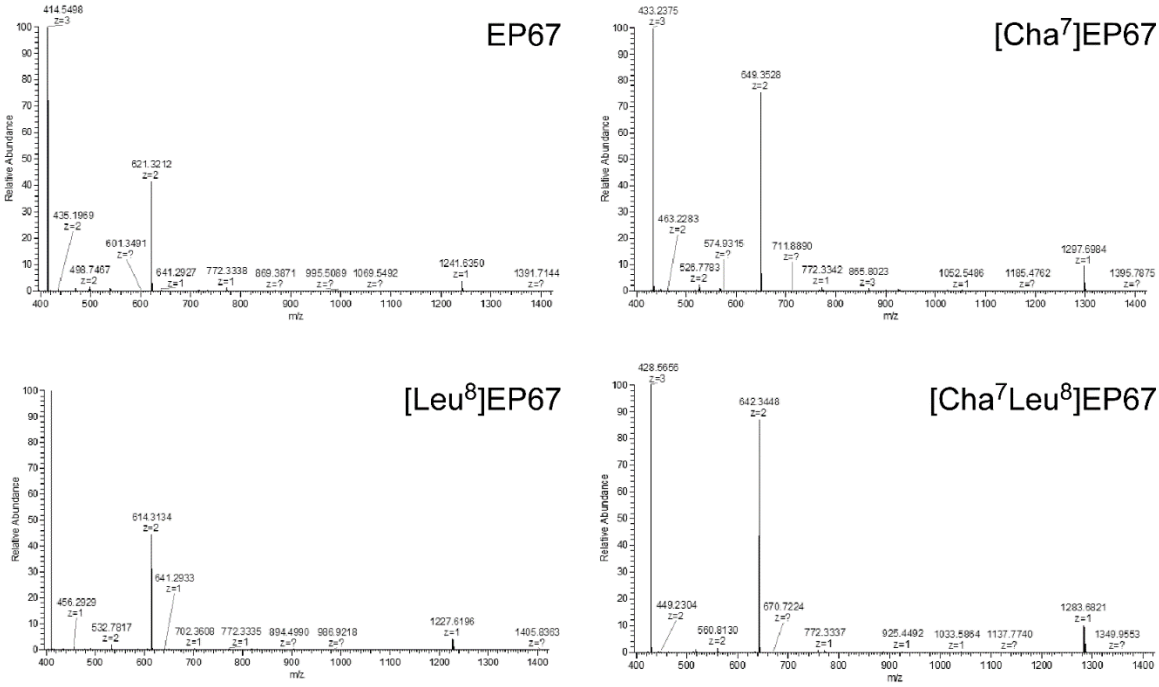


Figure 4.3 Mass chromatograms of EP67 and EP67 analogs. Cha = cyclohexylalanine. Masses were measured on an Orbitrap Fusion Lumos from ThermoScientific (Waltham MA) and with ≤ 0.5 ppm error. EP67: HRMS-ESI (*m/z*): $[M + H]^+$ calculated for ($C_{57}H_{88}N_{14}O_{15}S$ + H), 1241.6352; found 1241.6350. [Cha^7]EP67: HRMS-ESI (*m/z*): $[M + H]^+$ calculated for ($C_{61}H_{96}N_{14}O_{15}S$ + H), 1297.6978; found 1297.6984. [Leu^8]EP67: HRMS-ESI (*m/z*): $[M + H]^+$ calculated for ($C_{56}H_{86}N_{14}O_{15}S$ + H), 1227.6196; found 1227.6196. [Cha^{7}Leu^8]EP67: HRMS-ESI (*m/z*): $[M + H]^+$ calculated for ($C_{60}H_{94}N_{14}O_{15}S$ + H), 1283.6822; found 1283.6821.

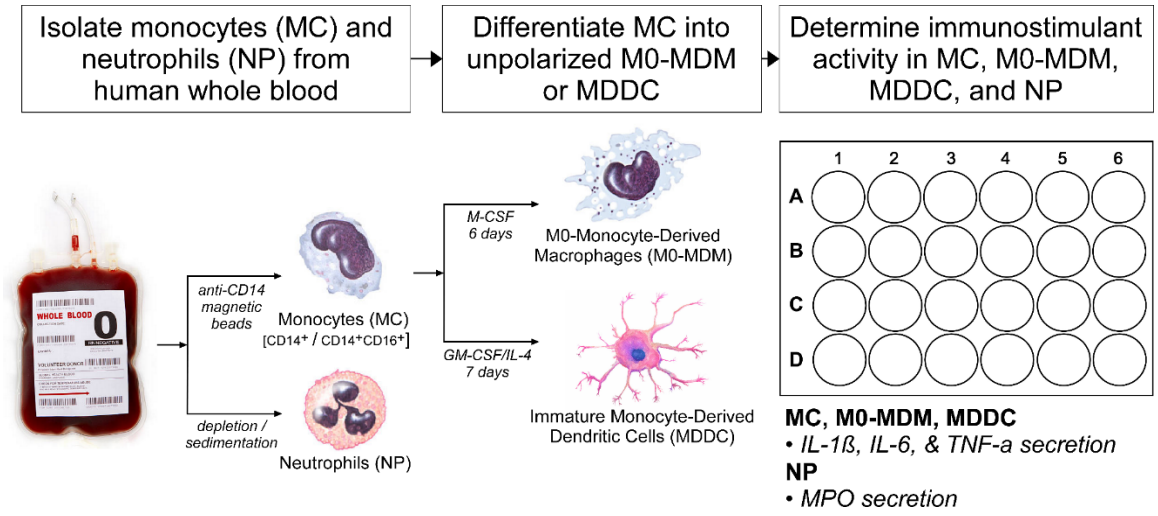


Figure 4.4 Experimental design for determining immunostimulant activity in human mononuclear phagocytes and neutrophils. Monocytes (MC) and neutrophils (NP) were isolated from the whole blood of healthy, human adult male donors (19 to 59 y.o.) using Miltenyi magnetic anti-CD14 MicroBeads and MACSxpress® Whole Blood Neutrophil Isolation Kits, respectively. Isolated NP were plated for 2 h before treatment, whereas isolated $CD14^+$/$CD14^+CD16^+$ MC were plated for 2 h before immunostimulant treatment or treated after differentiation into M0-monocyte-derived macrophages (M0-MDM) (human GM-CSF 6 d) or immature monocyte-derived dendritic cells (MDDC) (human GM-CSF/Il-4 for 7 d). For potency studies, cells were incubated with increasing concentrations of the indicated immunostimulant for 24 h and concentrations of IL-6 and TNF-α [MC, M0-MDM, MDDC] or myeloperoxidase (MPO) [NP] released into cell culture media was determined by ELISA. For kinetic studies, MC, M0-MDM, and MDDC were incubated with the EC_{50} of the indicated immunostimulant in the respective cell type and concentrations IL-6 and TNF-α released into cell culture media were determined after 6, 12, 24, and 48 h by ELISA.

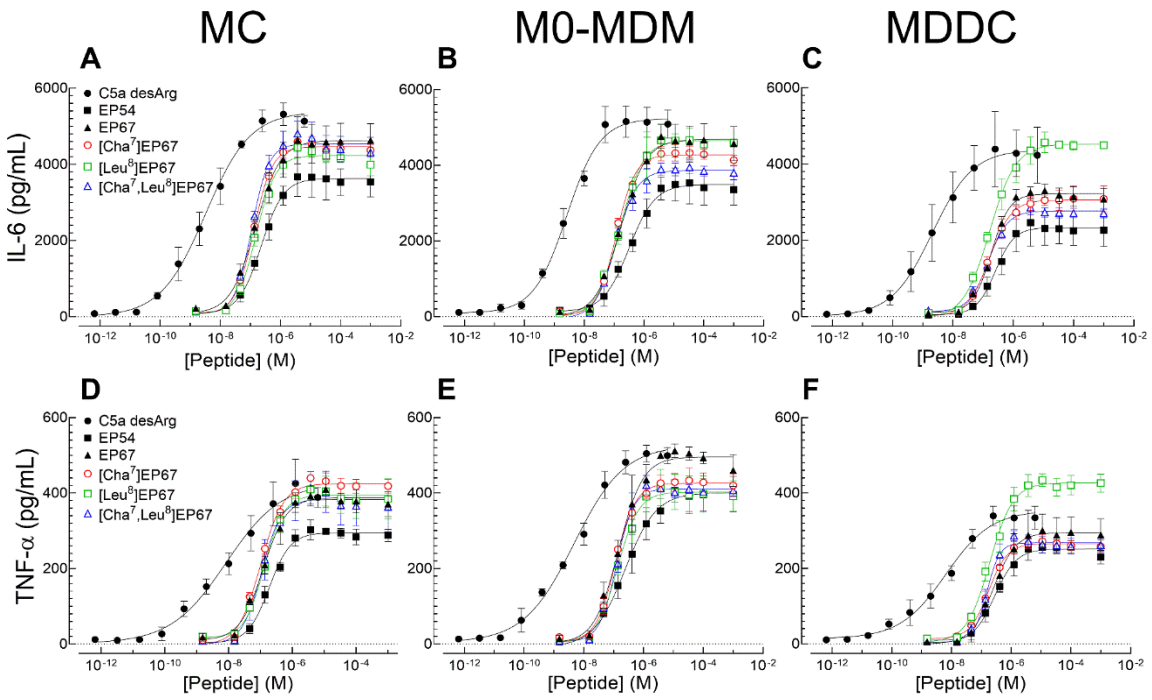
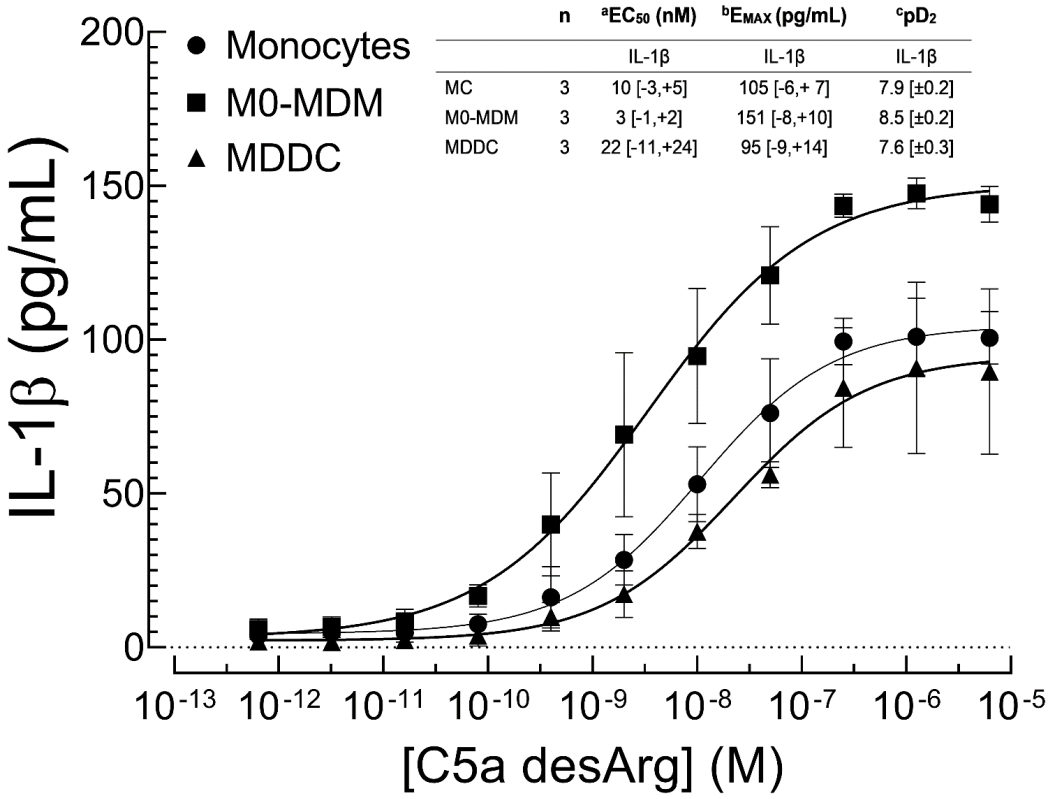


Figure 4.5 Dose response of IL-6 and TNF-α secretion from human monocytes, M0-monocyte-derived macrophages, and immature monocyte-derived dendritic cells after treatment with human C5a desArg, EP54, EP67, or EP67 analogs for 24 h. Human monocytes (MC), unpolarized M0-monocyte-derived macrophages (M0-MDM), and immature monocyte-derived dendritic cells (MDDC) were prepared from the whole blood of healthy, young human adult male donors (Fig. 4.4). Cells were then treated with increasing concentrations of human C5a desArg pooled from the blood of multiple donors (closed black circles), 1st-generation EP54 (closed black squares), 2nd-generation EP67 (closed black triangles), [Cha^{7}]EP67 (open red circles), [Leu^{8}]EP67 (open green squares), or [Cha^{7}, Leu^{8}]EP67 (open blue triangles). After 24 h, average concentrations (± SD) (n=2 replicates from 3 blood donors) of IL-6 or TNF-α secreted into the cell culture media of human monocytes (A & D), M0-MDM (B & E), or MDDC (C & F) were determined by ELISA and fit with a four-parameter dose-response curve where $Y=Min + (x^{HillSlope})*(Max-Min)/(x^{HillSlope} + EC_{50}{}^{HillSlope})$



	n	$^{a}EC_{50}$ (nM)	$^{b}E_{MAX}$ (pg/mL)	$^{c}pD_2$
		IL-1β	IL-1β	IL-1β
MC	3	10 [-3,+5]	105 [-6,+ 7]	7.9 [±0.2]
M0-MDM	3	3 [-1,+2]	151 [-8,+10]	8.5 [±0.2]
MDDC	3	22 [-11,+24]	95 [-9,+14]	7.6 [±0.3]

Figure 4.6 Dose response of IL-1β secretion from human monocytes, uncommitted M0-monocyte-derived macrophages, and immature monocyte-derived dendritic cells after treatment with human C5a desArg for 24 h. Human monocytes (MC), M0-monocyte-derived macrophages (M0-MDM), and immature monocyte-derived dendritic cells (MDDC) were prepared from the whole blood of healthy, young human adult male donors .Cells were then treated with increasing concentrations of human C5a desArg pooled from the blood of multiple donors. After 24 h, average concentrations (± SD) (n=2 replicates from 3 donors) of IL-1β in the cell culture media of human monocytes (black circles), M0-MDM (black squares), or MDDC (black triangles) were determined by ELISA and fit with a four-parameter dose-response curve where $Y=Min + (x^{HillSlope})*(Max-Min)/(x^{HillSlope} + EC_{50}^{HillSlope})$. IL-1β release was not observed from MC, M0-MDM, or MDDC after treatment with up to 1 mM of EP54, EP67, or EP67 analogs (not shown). Values calculated from dose response curves. n = number of healthy, young human adult male blood donors. $^{a}EC_{50}$ = average molar peptide concentration that stimulated 50% maximum secretion of indicated cytokine [±95%CI]. $^{b}E_{MAX}$ = average maximum mass concentration of secreted cytokine [±95%CI]. $^{c}pD_2$ = - log (EC_{50}) [±95% CI].

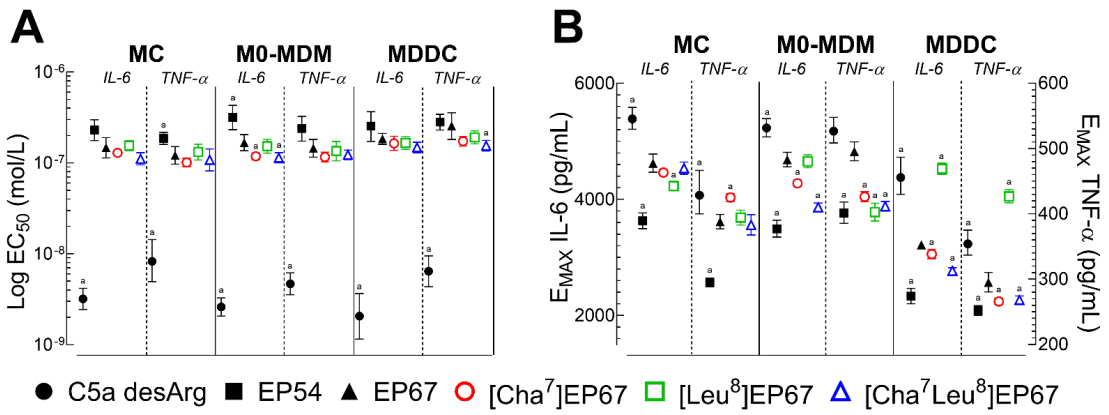


Figure 4.7 Comparison of potencies and efficacies for IL-6 and TNF-α secretion from human mononuclear phagocytes after treatment with human C5a desArg, EP54, EP67, or EP67 analogs for 24 h. Average (A) potencies (EC_{50}) and (B) efficacies (E_{MAX}) ±95% CI for IL-6 (left Y-axis) and TNF-α (right Y-axis) were calculated from dose response curves of IL-6 and TNF-α secretion from human monocytes (MC), M0-monocyte-derived macrophages (M0-MDM), and monocyte-derived dendritic cells (MDDC) after treatment for 24 h. 0.05 level of statistical difference vs. [a]EP67 (black triangles) for the indicated cytokine in the same cell type.

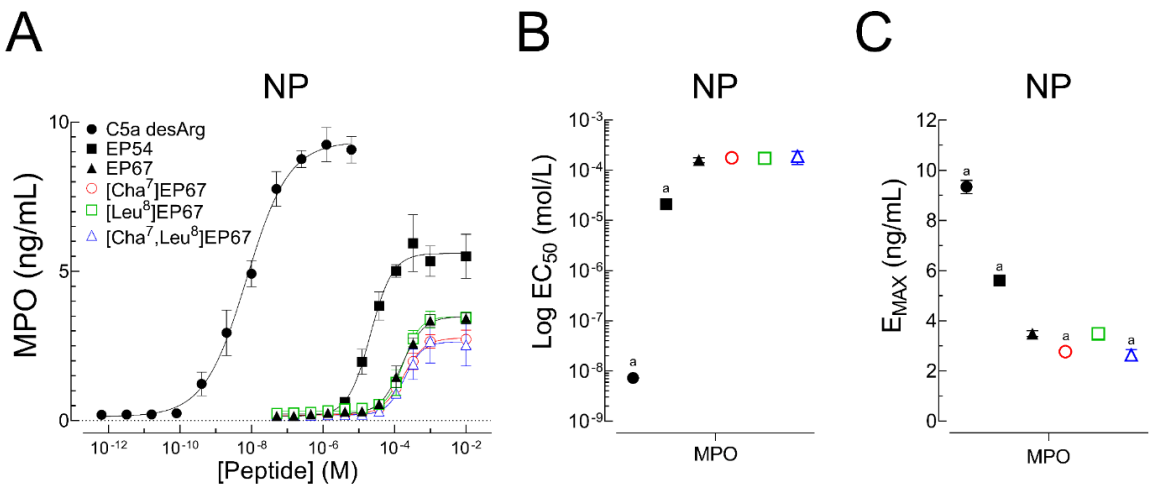


Figure 4.8 Dose response of myeloperoxidase release from human neutrophils after treatment with human C5a desArg, EP54, EP67, or EP67 analogs for 24 h. (A) Human neutrophils (NP) were prepared from the whole blood of healthy, young human adult male donors (18 to X y.o.) (Figure 4.4). NP were then treated with increasing concentrations of human C5a desArg pooled from the blood of multiple donors (black circles), 1st-generation EP54 (black squares), 2nd-generation EP67 (black triangles), [Cha^{7}]EP67 (open red circles), [Leu^{8}]EP67 (open green squares), or [Cha^{7}, Leu^{8}]EP67 (open blue triangles). After 24 h, average concentrations (± SD) (n=2 replicates from 3 blood donors) of myeloperoxidase (MPO) released into cell culture media were determined by ELISA and fit with a four-parameter dose-response curve where Y=Min + ($x^{HillSlope}$)*(Max-Min)/($x^{HillSlope}$ + $EC_{50}^{HillSlope}$). Average (B) potencies (Log EC_{50}) and (C) efficacies (E_{MAX}) ±95% CI for MPO secretion were calculated from the MPO dose response curves (A). ^{a}0.05 level of statistical difference vs. EP67.

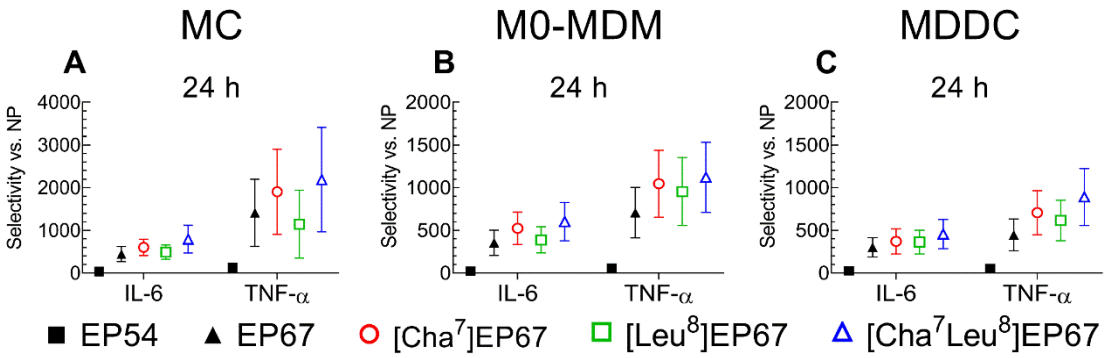


Figure 4.9. Comparison of selective activation of human mononuclear phagocytes vs. human neutrophils after treatment with human C5a desArg, EP54, EP67, or EP67 analogs for 24 h. Average selectivities ± propagated 95% CI for stimulating the secretion of IL-6 or TNF-α from human (A) MC, (B) M0-MDM, or (C) MDDC over secretion of MPO from human neutrophils (NP) were calculated from respective EC_{50} values after treatment for 24

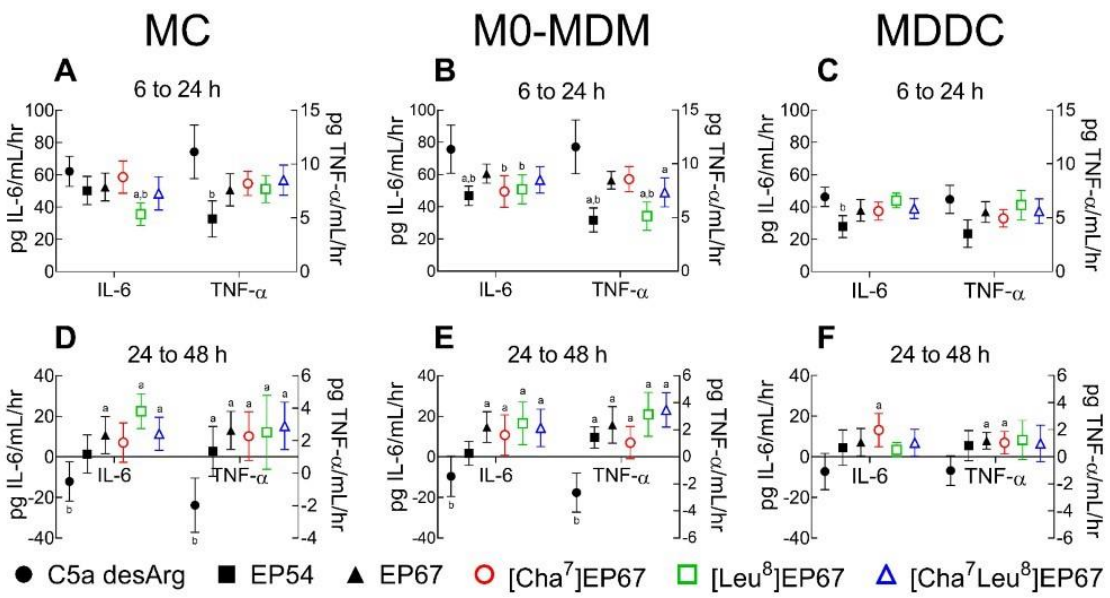


Figure 4.10 Comparison of IL-6 and TNF-α cytokine release rates from human monocytes, M0-monocyte-derived macrophages, and immature monocyte-derived dendritic cells after treatment with C5a desArg, EP54, EP67, or EP67 analogs. Human monocytes (MC), M0-monocyte-derived macrophages (M0-MDM), and immature monocyte-derived dendritic cells (MDDC) were prepared from the whole blood of healthy, young human adult male donors. Cell types were then treated with the calculated EC_{50} of human C5a desArg pooled from the blood of multiple donors (black closed circles), 1st-generation EP54 (black closed squares), 2nd-generation EP67 (black closed triangles), [Cha7]EP67 (red open circles), [Leu8]EP67 (green open squares), or [Cha7, Leu8]EP67 (blue open triangles). At the indicated time points, average concentrations (± SD) (n=2 replicates from 3 donors) of IL-6 or TNF-α released into cell culture media by human monocytes (A & D), M0-MDM (B & E), and MDDC (C & F) were determined by ELISA.

Peptide	Molecular mass (g/mol)	Amino Acid Sequence
C5a	10,400 (±1,000)	---$Ile^{65}Ser^{66}His^{67}Lys^{68}Asp^{69}Met^{70}Gln^{71}Leu^{72}Gly^{73}Arg^{74}$
C5a desArg	10,250 (±1,000)	---$Ile^{65}Ser^{66}His^{67}Lys^{68}Asp^{69}Met^{70}Gln^{71}Leu^{72}Gly^{73}$
EP54	1209.6	$Tyr^{01}Ser^{02}Phe^{03}Lys^{04}Pro^{05}Met^{06}Pro^{07}Leu^{08}$(d-Ala)$^{09}Arg^{10}$
EP67	1241.6	$Tyr^{01}Ser^{02}Phe^{03}Lys^{04}Asp^{05}Met^{06}Pro^{07}$(nme-Leu)^{08}(D-Ala)$^{09}Arg^{10}$
[Cha^{7}]EP67	1297.7	$Tyr^{01}Ser^{02}Phe^{03}Lys^{04}Asp^{05}Met^{06}$**Cha^{07}**(nme-Leu)^{08}(D-Ala)$^{09}Arg^{10}$
[Leu^{8}]EP67	1227.6	$Tyr^{01}Ser^{02}Phe^{03}Lys^{04}Asp^{05}Met^{06}Pro^{07}$**Leu^{08}**(D-Ala)$^{09}Arg^{10}$
[Cha^{7}, Leu^{8}]EP67	1283.7	$Tyr^{01}Ser^{02}Phe^{03}Lys^{04}Asp^{05}Met^{06}$**Cha^{07}Leu^{08}**(D-Ala)$^{09}Arg^{10}$

Table 4.1. Relevant sequences and molecular masses of C5a, C5a desArg, EP54, EP67, and EP67 analogs. Cha = cyclohexylalanine; nme-Leu = *N*-methyl leucine. Molecular masses of C5a and C5a desArg vary due to differences in glycosylation levels. C5a was not used in the current study because it is quickly converted to C5a desArg *in vivo*.

Peptide	[a]ACE-5-C18-300		[b]ACE-5-Phenyl-300		[c]ACE-5-CN-300	
	Retention time (min)	Purity (%)	Retention time (min)	Purity (%)	Retention time (min)	Purity (%)
EP54	24.4	86.2	24.9	87.2	23.8	87.2
EP67	27.4	96.9	27.4	96.1	22.3	94.4
[Cha^{7}]EP67	34.4	96.4	33.1	93.9	28.9	96.5
[Leu^{8}]EP67	26.1	99.1	25.1	99.0	20.7	99.7
[Cha^{7}Leu^{8}]EP67	34.5	97.4	31.8	95.5	28.2	99.2

Table 4.2. RP-HPLC Characteristics of EP54, EP67, and EP67 analogs. Cha = cyclohexylalanine. Retention time and purity were determined from RP-HPLC chromatograms using the indicated column. [a]ACE 5 C18-300 column (250 x 4.6 mm). [b]ACE 5 Phenyl-300 column (250 x 4.6 mm). [c]ACE 5 CN-300 column (250 x 4.6 mm).

Peptide	Calculated Mass ($[M+H]^+$)	Experimental Mass ($[M+H]^+$)	Error (ppm)
EP54	1209.6454	1209.6455	0.1
EP67	1241.6352	1241.6350	0.2
[Cha^7] EP67	1297.6978	1297.6984	0.5
[Leu^8] EP67	1227.6196	1227.6196	0.0
[Cha^7Leu^8] EP67	1283.6822	1283.6821	0.2

Table 4.3. Measured molecular masses of EP54, EP67, and EP67 analogs. Cha = cyclohexylalanine; nme-Leu = *N*-methyl leucine. Experimental masses determined from mass chromatograms**.**

Peptide	Asx	Ser	Ala	Met	Leu	Tyr	Phe	Lys	Arg	Pro
EP54		0.95 (1)	0.96 (1)	0.98 (1)	1.01 (1)	1.02 (1)	1.01 (1)	1.00 (1)	1.02 (1)	2.06 (2)
EP67	1.02 (1)	1.15 (1)	0.95 (1)	0.91 (1)		0.96 (1)	0.96 (1)	0.93 (1)	1.05 (1)	1.07 (1)
[Cha^{7}]EP67	1.01 (1)	0.99 (1)	0.95 (1)	1.01 (1)		1.01 (1)	1.02 (1)	1.00 (1)	1.01 (1)	
[Leu^{8}]EP67	0.99 (1)	0.98 (1)	0.95 (1)	1.03 (1)	1.01 (1)	0.99 (1)	1.01 (1)	1.00 (1)	1.01 (1)	1.03 (1)
[Cha^{7}Leu^{8}]EP67	1.00 (1)	0.99 (1)	0.96 (1)	1.02 (1)	1.02 (1)	1.00 (1)	1.02 (1)	1.00 (1)	1.01 (1)	

Table 4.4. Amino acid compositions of EP54, EP67, and EP67 analogs. Cha = cyclohexylalanine; nme-Leu = *N*-methyl leucine. Samples were subjected to vapor-phase hydrolysis in constant boiling 6M hydrochloric acid for 24 hours prior to being loaded on to a Hitachi 8800 Amino Acid Analyzer.

Peptide	n	[a]EC_{50} (nM)		[b]E_{MAX} (pg/mL)		[c]pD_2		[d]ΔC5a desArg		[e]%C5a desArg Potency	
A. MC		IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α
C5a desArg	3	3 [-0.7,+1][a]	8 [-3,+6][a]	5386 [-178,+197][a]	428 [-28,+38][a]	8.4 [±0.1]	8.1 [±0.2]	0	0	100	100
EP54	3	230 [-54,+68]	186 [-26,+31][a]	3629 [-133,+138][a]	295 [-7,+7][a]	6.6 [±0.1]	6.73 [±0.7]	1.9	1.3	1.4	4.4
EP67	3	147 [-34,+43]	121 [-24,+30]	4622 [-152,+159]	388 [-11,+11]	6.8 [±0.1]	6.9 [±0.1]	1.7	1.2	2.2	6.8
[Cha^7]EP67	3	129 [-10,+10]	101 [-10,+11]	4462 [-51,+51]	425 [-6,+6][a]	6.89 [±0.03]	6.99 [±0.05]	1.61	1.09	2.48	8.16
[Leu^8]EP67	3	155 [-19,+22]	131 [-24,+29]	4229 [-82,+84][a]	394 [-11,+11]	6.81 [±0.06]	6.88 [±0.09]	1.69	1.20	2.06	6.28
[Cha^7Leu^8] EP67	3	111 [-16,+18]	108 [-26,+34]	4536 [-105,+106]	376 [-15,+16][b]	6.96 [±0.7]	7 [±0.1]	1.5	1.1	2.9	7.7
B. M0-MDM		IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α
C5a desArg	3	2.6 [-0.5,+0.7][a]	5 [-1,+1][a]	5231 [-154,+161][a]	527 [-18,+21]	8.6 [±0.1]	8.3 [±0.1]	0	0	100	100
EP54	3	317 [-86,+112][a]	239 [-66,+86]	3493 [-142,+149][a]	401 [-16,+17][a]	6.5 [±0.1]	6.6 [±0.1]	2.1	1.7	0.89	1.9
EP67	3	167 [-31,+37]	145 [-29,+36]	4683 [-123,+128]	496 [-14,+15]	6.78 [±0.09]	6.8 [±0.1]	1.81	1.5	1.56	3.2
[Cha^7]EP67	3	118 [-10,+11][a]	116 [-14,+15]	4277 [-56,+56][a]	426 [-7,+8][a]	6.93 [±0.04]	6.9 [±0.6]	1.65	1.4	2.21	4.0
[Leu^8]EP67	3	153 [-25,+29]	135 [-30,+37]	4654 [-109,+112]	403 [-13,+14][a]	6.81 [±0.08]	6.9 [±0.1]	1.77	1.5	1.70	3.5
[Cha^7Leu^8] EP67	3	114 [-14,+16][a]	123 [-14,+15]	3864 [-73,+73][a]	411 [-7,+7][a]	6.94 [±0.06]	6.91 [±0.05]	1.64	1.42	2.28	3.78
C. MDDC		IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α
C5a desArg	3	2 [-0.9,+2][a]	6 [-2,+3][a]	4377 [-289,+349][a]	354 [-17,+21][a]	8.7 [±0.2]	8.2 [±0.2]	0	0	100	100
EP54	3	254 [-81,+112]	281 [-51,+61]	2331 [-130,+136][a]	252 [-7,+8][a]	6.6 [±0.2]	6.55 [±0.09]	2.1	1.64	0.8	2.28
EP67	3	184 [-24,+27]	255 [-74,+101]	3217 [-65,+67]	295 [-14,+15]	6.7 [±0.06]	6.6 [±0.1]	1.9	1.6	1.12	2.5
[Cha^7]EP67	3	164 [-27,+32]	173 [-20,+23]	3057 [-79,+83][a]	253 [-5,+5][a]	6.8 [±0.08]	6.76 [±0.05]	1.90	1.43	1.26	3.70
[Leu^8]EP67	3	165 [-25,+28]	191 [-29,+34]	4525 [-95,+98][a]	427 [-10,+10][a]	6.8 [±0.07]	6.72 [±0.07]	1.90	1.47	1.25	3.40
[Cha^7Leu^8] EP67	3	149 [-18,+21]	155 [-20,+22][a]	2770 [-50,+51][a]	268 [-7,+6][a]	6.8 [±0.06]	6.81 [±0.06]	1.86	1.38	1.387	4.13

Table 4.5 Potencies and E_{MAX} of IL-6 and TNF-α secretion from human monocytes, M0-monocyte-derived macrophages, and monocyte-derived dendritic cells after treatment with human C5a desArg, EP54, EP67, or EP67 analogs for 24 h. Values were calculated from dose response curves for (**A.**) human monocytes (MC), (**B.**) M0-monocyte-derived macrophages (M0-MDM), and (**C.**) monocyte-derived dendritic cells (MDDC) (**Figure 2**). Cha = cyclohexylalanine. n = number of healthy, young human adult male blood donors. [a]EC_{50} = average molar peptide concentration that stimulated 50% maximum secretion of the indicated cytokine [±95%CI]. [b]E_{MAX} = average maximum mass concentration of secreted cytokine [±95%CI]. [c]pD_2 = - log (EC_{50}) [±95% CI]. [d]ΔC5a desArg = pD_2 (C5a desArg) - pD_2 (peptide). [e]%C5a desArg Potency = antilog (-ΔC5a desArg) x 100.

Peptide	n	[a]EC_{50} (μM)	[b]E_{MAX} (ng/mL)	[c]pD_2	[d]ΔC5a desArg	[e]%C5a desArg Potency
NP		MPO	MPO	MPO	MPO	MPO
C5a desArg	3	*0.007 [±0.001]	*9.3 [±0.3]	8.14 [±0.08]	0	100
EP54	3	*21 [-3, +4]	*5.6 [±0.2]	4.67 [±0.08]	3.46	0.0343
EP67	3	160 [-17,+20]	3.5 [±0.1]	3.79 [±0.05]	4.34	0.00453
[Cha^7]EP67		177 [-21,+24]]	*2.8 [±0.1]	3.75 [±0.05]	4.39	0.00409
[Leu^8]EP67		173 [-19,+21]	3.4 [±0.1]	3.76 [±0.05]	4.38	0.00419
[Cha^7Leu^8]EP67	3	190 [-46,+61]	*2.6 [±0.2]	3.7 [±0.1]	4.4	0.0038

Table 4.6 Potencies and E_{MAX} of myeloperoxidase secretion from human neutrophils after treatment with human C5a desArg, EP54, EP67, or EP67 analogs for 24 h. Values were calculated from dose response curves of human neutrophils (NP). Cha = cyclohexylalanine. n = number of healthy, young human adult male blood donors. [a]EC_{50} = average molar concentration that stimulated 50% maximum secretion of myeloperoxidase (MPO) [±95%CI]. [b]E_{MAX} = average maximum mass concentration of secreted MPO [±95%CI]. [c]pD_2 = - log (EC_{50}) [±95% CI]. [d]ΔC5a desArg = pD_2 (C5a desArg) - pD_2 (peptide). [e]%C5a desArg Potency = antilog (-ΔC5a desArg) x 100. *0.05 level of statistical difference vs. EP67.

Peptide	n	[a]MC vs. NP Selectivity		[b]M0-MDM vs. NP Selectivity		[c]MDDC vs. NP Selectivity	
		IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α
C5a desArg	3	1	1	1	1	1	1
EP54	3	37 [±15]	126 [±70]	23 [±10]	59 [±25]	29 [±17]	52 [±21]
EP67	3	447 [±175]	1413 [±789]	355 [±148]	708 [±295]	302 [±113]	447 [±186]
[Cha^{7}]EP67	3	603 [±195]	1905 [±995]	525 [±189]	1047 [±392]	372 [±146]	708 [±259]
[Leu^{8}]EP67	3	490 [±169]	1145 [±795]	389 [±153]	955 [±398]	363 [±139]	617 [±236]
[Cha^{7}Leu^{8}]EP67	3	794 [±324]	2188 [±1223]	603 [±225]	1122 [±411]	457 [±171]	891 [±334]

Table 4.7 Selectivities of human C5a desArg, EP54, EP67, and EP67 analogs for the activation of human monocytes, M0-monocyte-derived macrophages, and monocyte-derived dendritic cells vs. human neutrophils. Average selectivities [± propagated 95% CI] were calculated. Cha = cyclohexylalanine. n = number of healthy, young human adult male blood donors. [a]MC vs. NP Selectivity = [antilog (-ΔC5a desArg MC) - (-ΔC5a desArg NP)], [b]M0-MDM vs. NP selectivity = [antilog (-ΔC5a desArg M0-MDM) - (-ΔC5a desArg NP)], and [c] MDDC vs. NP selectivity = [antilog (-ΔC5a desArg MDDC) - (-ΔC5a desArg NP)] where ΔC5a desArg = pD_2 (C5a desArg) - pD_2 (peptide) and pD_2 = - log (EC_{50}[M]) in the respective cell types.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5.1 CONCLUSION

The decapeptide EP67 is a novel host-derived immunostimulants based on the C-terminal of C5a that selectively activates phagocytes over neutrophils. In contrast to C5a65-74 from the C5a parent molecule, EP67 has topographical features that are well accommodated by C5aRs expressed on phagocytes-monocytes, macrophages, and dendritic cells, but not C5aRs neutrophils. Thus, EP67 induces host innate immune responses against normal and resistant infections via engagement of C5aR-bearing phagocytes with little/no inflammatory side-effects associated with the direct activation of neutrophils.

The Pro residue at position 7 (Pro7) is critical for the potency and selectivity of EP67. It was placed in this position to force an extended conformation at the carbonyl carbon between the ω and ψ bonds in the Met backbone to its N-terminus, a feature that was found to be biologically important from SAR studies with the previous analog of EP67, EP54. The Pro residue, however, undergoes cis/trans isomerization that leads to conformers of EP67 in solution, making it unclear whether cis/trans isomerization is important to the biological properties of EP67 or if one conformer is more effective than the other. What is clear is that the conformational influence of Pro at this position is important for the activity and bio-selectivity of EP67**.** Thus, the goals of this project were to (i.) develop a clinically

relevant, high-throughput assay for screening immunostimulant activity in primary human mononuclear phagocytes (monocytes, unpolarized (M0)-monocyte-derived macrophages, monocyte-derived dendritic cells) and neutrophils and (ii.) determine whether replacing Pro^{7} with cyclohexylalanine and/or nme-Leu^{8} with leucine adversely affects EP67 potency and efficacy in human mononuclear phagocytes and selective activation vs. human neutrophils.

In this project, we established a more clinically relevant, high throughput assay to determine immunostimulants activity in human monocytes, unpolarized monocytes-derived macrophages, monocytes-derived dendritic cells, and human neutrophils. Using this assay, we found that replacing Pro^{7} with cyclohexylalanine and/or nme-Leu^{8} with native Leu selectively affects EP67 potency and efficacy in human mononuclear phagocytes without affecting selective activation over human neutrophils. Thus, replacing Pro^{7} and nme-Leu^{8} with amino acids expected to induce similar structure changes is a suitable approach for generating future analogs of EP67.

5.2 FUTURE DIRCTIONS

Using our assay, we will continue screening for more EP67 analogs, and we expect that at least one analog with a 5 to 10-fold higher C5aR potency and bio-selectivity than EP67. Once identified, these analogs will be assessed for their

effectiveness in inducing innate immune responses against resistant bacterial (MRSA) infections. Briefly, we will demonstrate the therapeutic efficacy of EP67/analog in inducing host-innate immune responses against local and systemic methicillin-resistant Staphylococcus aureus (MRSA) infections in the human-relevant porcine model. We will also evaluate both the prophylactic and therapeutic efficacy of EP67/analog against these MRSA infections and will characterize the molecular and cellular elements of innate immune outcomes responsible MRSA reduction/elimination. We will establish the duration of the EP67-mediated protective effects to MRSA infections in the skin, blood, and kidney in our porcine model.

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